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Haemophilus parainfluenzae in Bronchiectasis

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Declaration

The work described in this thesis was performed by myself, the thesis was written by myself.

The work was undertaken in the Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh. And the Division of Pathway Medicine, the Chancellor's Building, University of Edinburgh.

This work has not previously been submitted for a higher degree or other professional qualification.

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Abstract

Introduction

Bronchiectasis is a chronic airway condition with permanently dilated airways, dysfunctional cilia and overproduction of mucus as main clinical symptoms, and in bronchiectasis there is a continuous cycle involving bacterial infection, host immune response and airway damage (Cole, 1986). More than 70% of bronchiectasis patients are chronically colonised with potential pathogens (Angrill *et al.*, 2002). The *Haemophilus* species is the most commonly found bacteria in bronchiectasis (Chalmers *et al.*, 2012), with *Haemophilus influenzae* (*H. influenzae*) being considered a pathogen and *Haemophilus parainfluenzae* (*H. parainfluenzae*) a commensal bacteria. However, *H. parainfluenzae* have been found more and more frequently associated with lower airway diseases, with further data suggesting it can also play a pathogenic role. Up to date, there is limited research about the role of *H. parainfluenzae* in bronchiectasis. This study will investigate the potential pathogenicity of *H. parainfluenzae* in bronchiectasis on its own and when combined with *Pseudomonas aeruginosa* (*P. aeruginosa*), the second most frequent organism isolated in bronchiectasis. We hypothesised that: *H. parainfluenzae* are common in bronchiectasis, and in high bacterial loads, it can play a similar pathogenic role as *H. influenzae*; *H. parainfluenzae* can induce a stronger inflammatory response when combined with *P. aeruginosa*.

Main results

1. *H. parainfluenzae* are commonly found in bronchiectasis patient's lower airways, but it is seldom reported

Of the 140 patient sputum samples that were previously found to be infected with *Haemophilus* species, 40% of the patients presented *H. parainfluenzae* and 47% *H. influenzae*. Another 51 were unselected bronchiectasis patients' sputum samples were processed both in our laboratory and an NHS microbiology laboratory. 18% of these patients presented *H. parainfluenzae* in their samples according to our laboratory, but none according to the NHS laboratory.

2. *H. parainfluenzae* have similar clinical relevance as *H. influenzae* in patients with clinically stable bronchiectasis

From those previously mentioned 140 bronchiectasis patients, 63 had their serum and sputum inflammatory markers compared. 24 of them had *H. parainfluenzae* and 39 *H. influenzae*. There was a similar bronchiectasis disease severity index (BSI) score between these two groups. There was similar systemic inflammation, with no significant differences in white cell counts (WCC), erythrocyte sedimentation rate (ESR), C-reactive protein（CRP), intercellular adhesion molecule 1 (ICAM-1) or Myeloperoxidase (MPO). These results suggest that *H. parainfluenzae* and *H. influenzae* have similar clinical relevance in bronchiectasis.

3. *H. parainfluenzae* in high bacterial loads can stimulate significant inflammatory responses from epithelial cells

H. parainfluenzae (at a concentration of 10^7 cfu/mL) can stimulate a range of inflammatory responses comparable to those of *H. influenzae* (10^7 cfu/mL) and *P. aeruginosa* (10^7 cfu/mL) on 16HBE cells, NHBE cells and bronchiectasis patients' nasal epithelial cells.

3.1 Inflammatory responses from human bronchial epithelial cell line (16HBE)

H. parainfluenzae (10^7 cfu/mL) can trigger significant interleukin 8 (CXCL8, p=0.001); chemokine ligand 1 (CXCL1, p=0.004); ICAM-1(p=0.004) and lipocalin 2 (LCN2, p=0.04) production from 16HBE cells compared to the control group after 8 hours. There was no significantly difference between 10^7 cfu/ml *H. parainfluenzae*, *H. influenzae* and *P. aeruginosa*.

3.2 Inflammatory responses from normal human bronchial epithelial (NHBE) cells and bronchiectasis patients' nasal epithelial cells

H. parainfluenzae (10^7 cfu/mL) can trigger significantly more CXCL8, CXCL1 and ICAM-1 production in NHBE cells (p-values are 0.02, 0.033 and 0.02 respectively) and patients' nasal epithelial cells (p-values are 0.038, 0.0005 and 0.0001 respectively) compared to the control group.

4. Poly-bacterial infection reduces 16HBE cells' inflammatory response compared to single bacterial infection

When 16HBE cells were co-cultured with *H. parainfluenzae* (10^5 cfu/mL) and *P. aeruginosa* (10^5 cfu/mL) for 8 hours, there was a significant decrease in CXCL8, CXCL1 and LCN2 production by 16HBE cells compared to cells that were cultured with only *P. aeruginosa* (10^5 cfu/mL), p-values are 0.0007, 0.0032 and 0.04 respectively.

5. Indirect interaction between *H. parainfluenzae* and *P. aeruginosa* may lead to the change observed in inflammatory response from previous result

When 16HBE cells were co-cultured with heat-treated *H. parainfluenzae* (10^5 cfu/mL) and *P. aeruginosa* (10^5 cfu/mL) for 8 hours, there was no significant decrease in the co-infection group (CXCL8 p=0.16, CXCL1 p=0.2), which suggests that the previously observed reductive effect requires living bacteria. Therefore, this reduction is likely to be due to bacterial interaction between *H. parainfluenzae* and *P. aeruginosa*.

When 16HBE cells and *H. parainfluenzae* (10^5 cfu/mL) (n=6) were cultured in filtered media that had cultured *P. aeruginosa* (n=6) for 8 hours, there was a significant decrease of CXCL8 and CXCL1 levels compared to cells cultured with *H. parainfluenzae* in media that had not cultured *P. aeruginosa* (CXCL8 p=0.002, CXCL1 p=0.027. This suggests that *P. aeruginosa* induced media conditions may play a role in this observed inhibition effect. Later experiments revealed that *P. aeruginosa* can promote the growth of *H. parainfluenzae*.

Conclusion

H. parainfluenzae are commonly found in bronchiectasis patient's lower airways, and it is under-reported in bronchiectasis. Patients with *H. parainfluenzae* in their lower airways have similar sputum and serum inflammation compared to patients with *H. influenzae*. *H. parainfluenzae* can stimulate significant inflammatory responses from epithelial cells.

Poly-microbial (*H. parainfluenzae* with *P. aeruginosa*) infection reduces 16HBE cell inflammatory response (CXCL8, CXCL1 and LCN2) compared to single bacterial

infection. The media cultured with *P. aeruginosa* can reduce cellular inflammatory response when co-cultured with *H. parainfluenzae*. The underlying mechanism underpinning this merit further study.

Abbreviations

Haemophilus influenzae	*H. influenzae*
Haemophilus parainfluenzae	*H. parainfluenzae*
Pseudomonas aeruginosa	*P. aeruginosa*
Non-type-able H. influenzae	NTHi
White cell counts	WCC
Erythrocyte sedimentation rate	ESR
C-reactive protein	CRP
Intercellular adhesion molecule 1	ICAM-1
Myeloperoxidase	MPO
Human bronchial epithelial cell	16HBE
Normal human bronchial cells	NHBE
Pyocyanin	PCN
Interleukin 8	CXCL8
Chemokine ligand 1	CXCL1
Lipocalin 2	LCN2
Optical Density	OD
Chronic obstructive pulmonary disease	COPD
Connective tissue disorder	CTD
Allergic bronchopulmonary aspergillosis	ABPA
Staphylococcus aureus	*S. aureus*
Moraxella catarrhalis	*M. catarrhalis*
Streptococcus pneumoniae	*S. pneumoniae*
Hemin	X

Nicotinamide adenine dinucleotide	V
Hemin and nicotinamide adenine dinucleotide	XV
Cystic fibrosis	CF
Haemophilus influenzae type B	Hib
Haemophilus haemolyticus	*H. haemolyticus*
Enzyme-linked immunosorbent assay	ELISA
Sodium dodecyl sulphate–polyacrylamide gel electrophoresis	SDS-PAGE
Immunoglobulin G	IgG
Immunoglobulin A nephropathy	IgAN
Mitogen-activated kinase phosphatase 1	MKP-1
Lipopolysaccharides	LPS
Lipooligosaccharide	LOS
Interleukin 6	IL6
Tumour necrosis factor alpha	TNFα
Extensively drug-resistant	XDR
Toll-like receptor 4	TLR4
Transforming growth factor-b–associated kinase-1	TAK1
Myeloid differentiation primary response 88	MyD88
Nuclear factor-κB	NF-κB
Blood agar	BA
Chocolate blood agar	CBA
Interleukin 1 beta	IL1β
Escherichia coli	*E. coli*
Streptococcus gordonii	*S. gordonii*
Bronchiectasis severity index	BSI

Body-mass index	BMI
Pseudomonas isolation agar	PIA
H. influenzae specific protein D	*Hi hpd*
[Cu, Zn]-superoxide dismutase C	*sodC*
H. haemolyticus specific *hpd* genes	*Hh hpd*
Brain heart infusion broth	BHI broth
Fuculose Kinase	*fucK*
Bovine serum albumin	BSA
Phosphate Buffered Saline	PBS
Tetramethylbenzidine	TMB
Dulbecco's Modified Eagle Medium	DMEM
Lactate dehydrogenase	LDH
Inflammatory bowel disease	IBD
Tuberculosis	TB
Minimum	min
Maximum	max
Angiopoietin 1	ANG 1
Basic fibroblast growth factor	FGF-2
Hepatocyte growth factor	HGF
Leukemia inhibitory factor	LIF
Chemokine ligand 20	CCL20
Granulocyte-macrophage colony-stimulating factor	GM-csf
Urokinase receptor	uPAR
Interleukin 24	IL24
Interquartile range	IQR

Mixed normal flora	MNF
Pulsed-field gel electrophoresis	PFGE

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Chapter 1

Introduction

Chapter 1: Introduction

1.1 What is bronchiectasis?

Bronchiectasis is a chronic condition in which the lower airways become abnormally dilated; patients suffer from persistent bacterial infection and recurrent exacerbations. Common symptoms in bronchiectasis include daily cough, excessive sputum production and breathlessness. Microbiological results show more than 70% of patients are chronically colonized with a wide range of bacteria and present an excessive neutrophilic inflammation (Angrill *et al.*, 2001).

1.2 Epidemiology

Bronchiectasis was first described in 1819 as a rather rare phenomenon and for a long period of time it was called an 'orphan disease'. Since the beginning of the 21st century, with the more common use of high resolution computed tomography in the diagnosis of bronchiectasis (Chalmers and Hill, 2013), more and more data suggests that bronchiectasis is much more common than previously reported, and prevalence is quickly increasing since recent years (Jennifer K. Quint, et, 2016). In the UK, in 2013, the prevalence of bronchiectasis in women was reported at 566.1 per 100, 000, compared to the 350.5 per 100, 000 in 2004. In men, in 2013, there were 485.5 per 100, 000 reported, compared to the 301.2 per 100, 000 in 2004 (Jennifer K. Quint, et, 2016). Bronchiectasis is more common in people older than 40 years old, with more than 50% being over 65 years old (Jennifer K. Quint, et, 2016).

Bronchiectasis is associated with a markedly increased mortality compared to general population. In England and Wales, the mortality rate in women and men with bronchiectasis (n=11862) has been researched. In women, the age-adjusted mortality rate for bronchiectasis population was 1437.7 per 100,000, and for general population 635.9 per 100,000. In men, the age-adjusted mortality rate for the bronchiectasis population was 1914.6 per 100,000 and for the general population 895.2 per 100,000 (Jennifer K. Quint, et, 2016).

1.3 Aetiology

A considerable number of disorders are associated to bronchiectasis, with different possible aetiologies contributing to the progression of the disease. Post-infection, chronic obstructive pulmonary disease (COPD), connective tissue disorder (CTD), ciliary dysfunction and allergic bronchopulmonary aspergillosis (ABPA) are the most common known aetiologies (McDonnell *et al.*, 2016). In nearly half of the cases the underlying cause can not be identified. Identifying a specific aetiology may benefit patients with more specific treatments (Gao *et al.*, 2016).

1.4 Pathophysiology

It has been 200 hundred years since bronchiectasis was first described, but our understanding about it is still poor (Goeminne and Soyza, 2016). As previously mentioned, a lot of bronchiectasis cases occur post-infection. Bacterial infection contributes to lung damage directly by releasing toxins that can impair airway function, and indirectly by over stimulating the host's immune response. The immune response is a protective response from the body to harmful stimuli such as pathogens, and it can include the release of neutrophils, lymphocytes and macrophages into the bronchial lumen (Simpson *et al.*, 2007). These immune cells all work towards the elimination of pathogens. However, in bronchiectasis there is excessive neutrophilic inflammation, which means either an overproduction of neutrophils or an inefficient neutrophil clearance by the airway after phagocytosis (Boyton and Altmann, 2016)(Bedi *et al.*, 2018). Neutrophilic products can also reduce epithelial cilia beat frequency and cause mucous gland hypersecretion, which negatively affects mucociliary clearance (Amitani *et al.*, 1991). Neutrophils release chemicals such as elastase, which facilitates bacteria-killing, but can also damage bronchial elastin and other supporting lung structures, which leads to permanent dilatation of the bronchi (Amitani *et al.*, 1991; Margaroli *et al.*, 2019). Damaged bronchi contribute to pooling of mucus, which supports continued bacterial infection. In 1984, Cole described a 'vicious circle' in bronchiectasis, which pointed out the continuous circle of bacterial infection, immune

response and airway damage (Cole, 1986). A lot of research in bronchiectasis has aimed at breaking this cycle (Figure 1).

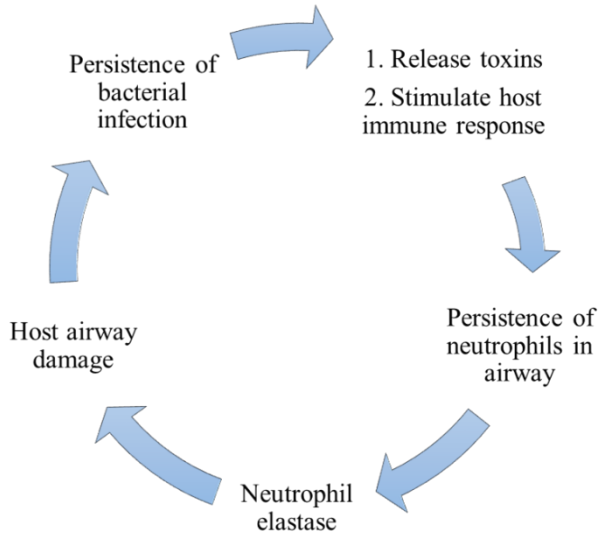


Figure 1. **The 'vicious circle' in bronchiectasis described by Cole** (Cole, 1986)

1.5 Bacterial infection in bronchiectasis

The lung is continuously exposed to a large number of bacteria; excessive airway neutrophilic inflammation leads to damage to the bronchial wall and, paradoxically, promotes more airway inflammation and bacterial infection, creating a vicious cycle (Cole, 1986). The bacterial infection is a crucial part of this circle.

In healthy lungs, infection is usually caused by microbial immigration (micro aspiration, inhalation of bacteria and direct mucosal dispersion) and clearance by microbial elimination (cough, mucociliary clearance and innate and adaptive host defences). In diseased lungs, bronchial infection is usually driven by regional bacterial growth and condition changes such as: pH value, temperature, oxygen tension, nutrient availability, local microbial competition, epithelial cell interactions, activation of inflammatory cells and concentration of inflammatory cells (Dickson, Martinez, & Huffnagle, 2014) (Dickson et al., 2014). In bronchiectasis, patients usually have permanently damaged and dilated airways with fewer cilia and more mucus (Rademacher & Welte, n.d.); mucus production results in decrease of oxygen tension (Worlitzsch et al., 2002) and an increase of temperature (Schmidt et al., 2014), which selectively contributes to bacterial growth. The inflammatory response continuously

creates a selection pressure across the bacterial community. This pressure results in a disordered microbiome, which provokes further airway inflammation. If the host fails to clear the bacteria and the bacterial infection persists, the inflammatory response becomes chronic.

The conventional view about bronchiectasis' bacterial infection has long been limited to a few specific pathogens (B.Rogers, 2018). However, more recent studies have pointed out that bronchiectasis airways are colonised with complex microbial communities. A large percentage of bacteria in this community are upper airway opportunistic pathogens, which migrate to colonise the lower airways (Dickson and Huffnagle, 2015). The development of technology has changed our understanding of bacteria; previously under-reported bacteria have been found in bronchiectasis airways nowadays, and the widespread use of vaccination and antibiotics are changing the prevalence of bacteria (King *et al.*, 2007). It is important to look outside the currently known pathogen model and explore more factors that may not have been included in the past.

1.6 Microbiology

Knowledge of lower airway microbiology is a very important in bronchiectasis treatment, in particular for future chest infections. Antibiotic selection is based on results from sputum microbiology tests and antibiotic sensitive tests. There is a defined group of bacteria that can be potentially pathogenic in bronchiectasis; a diagnostic is usually made based on this group. These species have been proven to be commonly found in bronchiectasis patients' lower airways and also clinically relevant for infection and colonisation. *Haemophilus influenzae* (*H. influenzae*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are the two most commonly found species, with *Staphylococcus aureus* (*S. aureus*), *Moraxella catarrhalis* (*M. catarrhalis*) and

Streptococcus pneumoniae (*S. pneumoniae*) also being widespread (Chalmers *et al.*, 2012) (Figure 2).

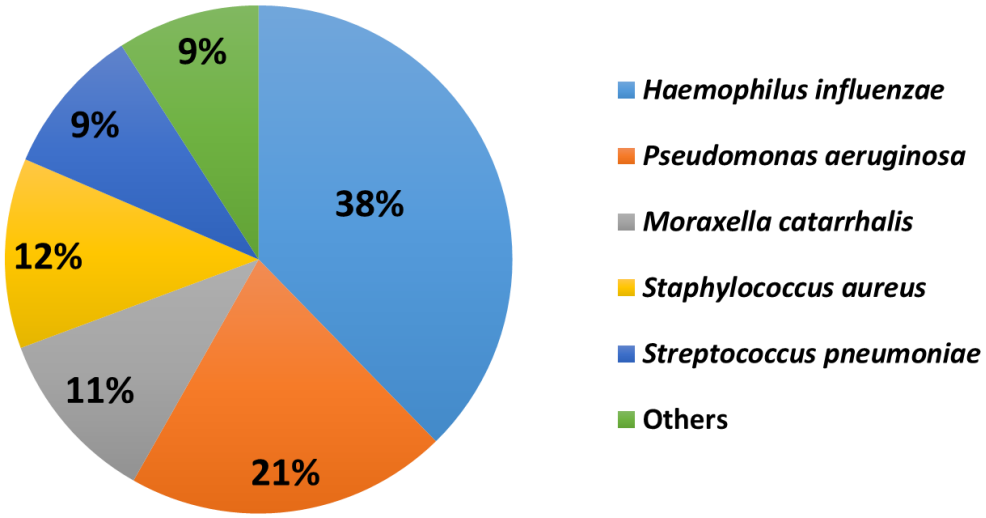


Figure 2. The main pathogens found in Bronchiectasis (Chalmers *et al.*, 2012)

1.7 *H. influenzae*

H. influenzae are aerobic Gram-negative bacteria, although it can also grow as a facultative anaerobe. *H. influenzae* are cultured on chocolate blood agar plates. The growth of *H. influenzae* requires X (hemin) and V (nicotinamide adenine dinucleotide) factors. They grow at 37°C in a CO_2 enriched environment. The colonies of *H. influenzae* on chocolate agar plates are convex, smooth, pale, grey or transparent in colour (Levine *et al.*, 1996).

There are capsulated *H. influenzae* and non-capsulated *H. influenzae*. Capsulated *H. influenzae* can be typed into type a, b, c, d, e and f, depending on their polysaccharide capsule (Brlrur and -Jourbml, 1952). Non-encapsulated *H. influenzae* can not be typed due to the lack of a capsule, and so they are also called as non-type-able *H. influenzae* (NTHi)(Davis *et al.*, 2011).

H. influenzae are important pathogen in other respiratory diseases such as otitis media, tonsillitis, COPD and cystic fibrosis (CF)(Erwin and Smith, 2007). Type b *H. influenzae* used to be the most common pathogenic one; however since the widespread use of *Haemophilus influenzae* type b (Hib) vaccination, the number of type b *H. influenzae* infection has significantly reduced, and NTHi prevalence has significantly increased (King *et al.*, 2007) (Van Eldere *et al.*, 2014).

While *H. influenzae* b is being neutralized by vaccination schemes, another *Haemophilus* species historically treated as a commensal bacterium in bronchiectasis is attracting more attention: *Haemophilus parainfluenzae* (*H. parainfluenzae*). While it used to be less common in the lower airways, *H. parainfluenzae* are now almost twice as frequent as *H. influenzae* (Ebbing and Robertson, 2015). It has also been found to have similar clinical relevance as *H. influenzae* in lower airway infection (Rhind *et al.*, 1985). Furthermore, *H. parainfluenzae* presents a higher multidrug resistance than *H. influenzae* (Scheifele and Fussel L, 1981; Ebbing and Robertson, 2015).

1.8 *H. parainfluenzae*

H. parainfluenzae are gram-negative coccobacillus, and it is a part of the normal human oropharyngeal and genitourinary microbiota. *H. parainfluenzae* and *H. influenzae* both belong to *Haemophilus* species. *Haemophilus* species are commonly isolated from all kinds of human specimen samples. They can be divided into three groups based on their nutritious needs: 1) *H. influenzae* group, X and V factor-dependent, i.e. *H. influenzae* and *Haemophilus haemolyticus* (*H. haemolyticus*); 2) *H. parainfluenzae* group, V factor-dependent, i.e. *H. parainfluenzae*; and 3) *Haemophilus ducreyi*, X factor-dependent.

H. parainfluenzae can be differentiated into eight subtypes depending on their production of three metabolic products: indole from tryptophan, urease and ornithine decarboxylase. Subtype I and II constitute most of the isolates of *H. parainfluenzae* from the oropharynx in controls (75%) and chronic bronchitis (90%). Subtype VI, VII and VIII are the least common ones (Taylor *et al.*, 1992).

H. parainfluenzae have historically been considered commensal bacteria in bronchiectasis. A paper published in 1976 indicated *H. parainfluenzae* as a commensal bacteria in lower airway disease, reporting that *H. parainfluenzae* were not as frequently isolated as *H. influenzae* from lower airways; it also suggested that *H. parainfluenzae* did not stimulate a significant immune response from the host (Smith *et al.*, 1976). However, with the development of technology, *H. parainfluenzae* have been found to be frequently isolated from lower airways (Ebbing and Robertson, 2015),

and more recent research suggest that *H. parainfluenzae* can trigger a significant innate and adaptive immune response from the host (Mitchell and Hill, 2009; Goleva *et al.*, 2013). The difficulty in identifying *H. parainfluenzae* could have led to misdiagnosis and an underestimated pathogenicity in the past.

1.9 Why *H. parainfluenzae* are important now

1.9.1 *H. parainfluenzae* are common in lower airways

H. parainfluenzae are ubiquitous on the human upper airways, representing more than 60% of the *Haemophilus* found in the human upper airways (Andrzejczuk et al., 2017). It has been isolated from the sputum of 70% of COPD patients(Simpson, Baines, et al., 2016), and it has also been found in corticosteroid-resistant asthma airways (Goleva et al., 2013).

A 15-year study about CF showed a significant increase in the prevalence of *H. parainfluenzae* in CF patients. Especially after 2010, the number of *H. parainfluenzae* was more than twice that of *H. influenzae*; while in 1998, the number of *H. parainfluenzae* was around half of *H. influenzae* (Figure 3). The significant increase of *H. parainfluenzae* in COPD was proposed to be because of the development of techniques to identify *H. parainfluenzae* and a higher awareness of *H. parainfluenzae* as a pathogen (Ebbing and Robertson, 2015).

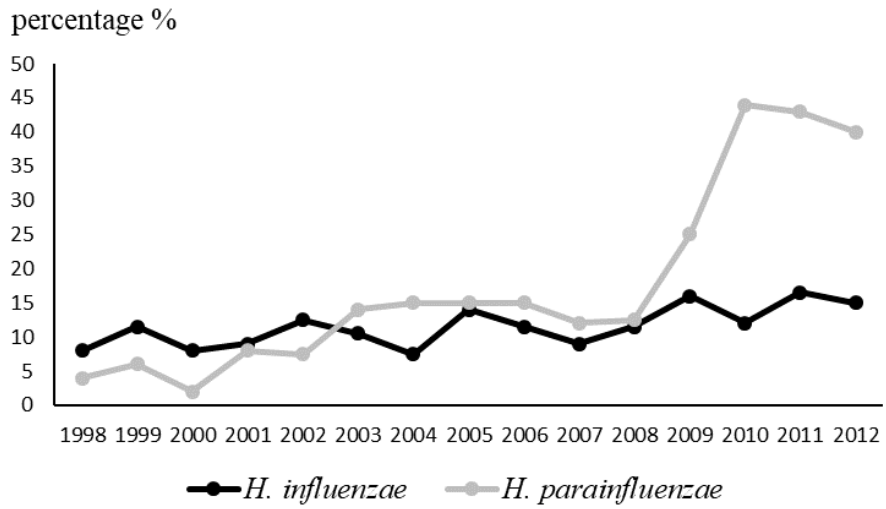


Figure 3. The prevalence of *H. influenzae* and *H. parainfluenzae* in CF from 1998 to 2012. (Ebbing and Robertson, 2015)

H. influenzae are considered one of the most common bacteria in bronchiectasis. However, there is contradictory data about the prevalence of *H. parainfluenzae* in bronchiectasis. A study of 144 patients showed that 10.4% had *H. parainfluenzae* and 9.7% *H. influenzae* (Guan *et al.*, 2015). Another study reported only 1 out of 70 patients with *H. parainfluenzae,* and 20% with *H. influenzae* (Purcell *et al.*, 2014). Another report followed 5 children that developed bronchiectasis after liver or kidney transplantation, in which 2 of them had *H. parainfluenzae* in the lower airways (Pijnenburg *et al.*, 2004).

1.9.2 *H. parainfluenzae* are associated with a variety of diseases

H. parainfluenzae have been related to a spectrum of diseases. It has been implicated in pneumonia, where the patient presented high concentrations of *H. parainfluenzae* in sputum, a high titre of *H. parainfluenzae* specific immunoglobulin M, as well as high serum erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (Pillai *et al.*, 2000). It has also been associated with acute otitis media (Cardines *et al.*, 2009), secondary bacteraemia (Kaushik *et al.*, 2015), endocarditis (Duzenli, Dwyer and Carey, 2017), urethritis (Hsu *et al.*, 2015), biliary tract infections (Frankard *et al.*, 2004), hepatic abscesses (Athreya, Hrastar and Khan, 2017) and meningitis (Cardines *et al.*, 2009).

1.9.3 *H. parainfluenzae* can be multidrug-resistant

Haemophilus species can be multidrug-resistant (Tristram, Jacobs and Appelbaum, 2007; Magiorakos *et al.*, 2012). *H. parainfluenzae* isolates producing TEM-1/ROB-1 beta-lactamases and with altered PBP3 (beta-lactamase negative ampicillin-resistant [BLNAR]) are being reported increasingly in recent years (Tinguely *et al.*, 2013). In 2011, a multidrug-resistant clinical isolate presenting resistance to quinolones, tetracycline and co-trimoxazole was reported in a prostatitis patient in Spain (Rodríguez-Martínez, López-Hernández and Pascual, 2011). Two cases of extensively drug-resistant *H. parainfluenzae* were found in Switzerland in 2013 (Tinguely *et al.*, 2013). These two *H. parainfluenzae* strains were non-susceptible to ampicillin, amoxicillin-clavulanate, cefotaxime, cefepime, meropenem, cefuroxime, azithromycin, ciprofloxacin, tetracycline, and chloramphenicol.

A study looked into *Haemophilus* species in sputum before and after 7 days of ampicillin treatment. They found that at day 0, 4 out of 5 patients only presented *H. influenzae*; after treatment, those *H. influenzae* were all replaced by *H. parainfluenzae* (Foweraker, Cooke and Hawkey, 1993).

In CF, *H. parainfluenzae* have been found more frequently associated with multidrug resistance than *H. influenzae*. With a higher resistance to ampicillin, amoxicillin and clavulanic acid, co-trimoxazole, rifampicin and cefotaxime than *H. influenzae* (Ebbing and Robertson, 2015).

In a throat culture survey, β-lactamase-producing *H. parainfluenzae* was detected in 192 out of 266 ambulatory children. Colonisation with β-lactamase-producing *H. parainfluenzae* was 26 times more frequent than with *H. influenzae* (Fussell, 2019). The study also proposed that *H. parainfluenzae* could be a vector for the spread of resistance genes to *H. influenzae*.

1.9.4 *H. parainfluenzae* can be pathogenic

One of the main reasons that *H. parainfluenzae* have been considered commensal bacteria is that, in the past, *H. parainfluenzae* was proven to be not virulent and could not stimulate host immune response (Smith *et al.*, 1976). However, modern studies highlight *H. parainfluenzae* as a potentially pathogenic organism. In the year of 2000, a group of researchers demonstrated the presence of a species-specific systemic immune response to *H. parainfluenzae* in colonised patients (Mitchell and Hill, 2000). In this study, they investigated serum and sputum immunoglobulin G from 10 bronchiectasis and 3 chronic bronchitis patients. These patients had *H. parainfluenzae* regularly isolated from their sputum. Using enzyme-linked immunosorbent assay (ELISA) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), they confirmed that those patients had significantly higher *H. parainfluenzae* specific immunoglobulin G (IgG) compared to healthy controls. There are also studies focused on immunoglobulin A nephropathy (IgAN) suggesting *H. parainfluenzae* antigens stimulate IgA antibodies, as well as tonsillar T and B lymphocytes in patients with IgAN (Suzuki *at al*, 1994; Suzuki *et al.*, 2000). This all suggests *H. parainfluenzae* can play a pathogenic role.

Another study indicated *H. parainfluenzae* can stimulate a significant pro-inflammatory response in asthma. *H. parainfluenzae* significantly activated macrophage p38 mitogen-activated protein kinase, which led to the production of interleukin 8 (CXCL8) as well as mitogen-activated kinase phosphatase 1 (MKP-1) mRNA. As a comparison, macrophages were co-cultured with *Prevotella melaninogenia*, which did not induce the same effect as *H. parainfluenzae* (Goleva *et al.*, 2013). *H. parainfluenzae* also stimulated CXCL8 production in A549 cells (Middleton, Dowling, *et al.*, 2003).

A small scale research (n=2) reported *H. parainfluenzae* could cause immediate slowing of nasal epithelial ciliary beat frequency, significantly more mucosal damage and significantly less ciliated and unciliated cells (Middleton, Dowling, *et al.*, 2003). In the same study, they found *H. parainfluenzae* had a tendency to adhere to the damaged epithelial area, which suggests that *H. parainfluenzae* may be more pathogenic in impaired airways rather than healthy ones. *H. parainfluenzae* adhered to a bronchial epithelial cell line to the same degree as NTHi (Pollard *et al.*, 2008).

1.10 What makes *H. parainfluenzae* virulent

1.10.1 Endotoxin of *H. parainfluenzae*

Lipopolysaccharides (LPS) are their main virulence factor. LPS located on the outer layer of the outer membrane; it is gram-negative bacteria's major cell surface component. It forms a chemical and physical barrier for bacteria. There is a low molecular weight form of LPS which is called lipooligosaccharide (LOS). *H. parainfluenzae* and *H. influenzae* both have LOS.

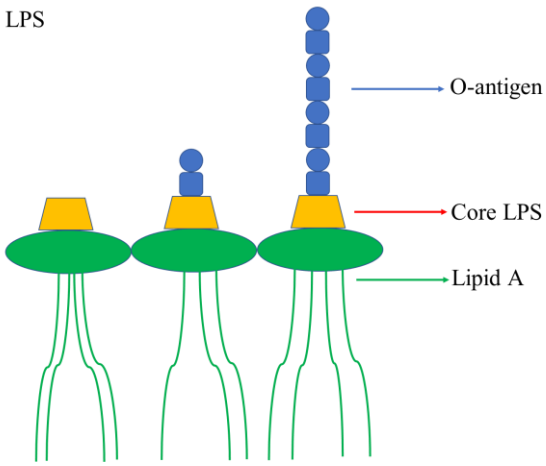


Figure 4. Model of LPS of gram-negative bacteria. Figure made based on Steimle Autenrieth and Frick (2016). ● ■ different sugar moieties; ∿ acyl chains of different length.

The structure of LOS can determine bacterial interactions with host cells. *H. parainfluenzae* and *H. influenzae* have similar LOS; they have identical lipid A and inner core (Young and Hood, 2013), however, there are some differences between these two close relatives. *H. parainfluenzae* have a reduced capacity for synthesis of outer core structures, including phosphocholine, sialic acid, digalactoside and Oacetyl groups; these are expressed in a phase variable manner, which suggest that there is a lack of phase variability in *H. parainfluenzae* LOS (Young and Hood, 2013). Those outer core peptides are involved in *H. influenzae* pathogenesis. It was found that the lack of outer core LOS in a mutant *H. parainfluenzae* led to reduced ability to adhere to the epithelial cell, which indicates that outer core LOS also play an important role in *H. parainfluenzae* colonisation. On the other hand, *H. parainfluenzae* LOS contains O-antigen, which is not found in *H. influenzae* LOS. O-antigen can contribute significantly to *H. parainfluenzae* resistance to the killing effect of human serum *in vitro* (Young *et al.*, 2013)(Vitiazeva *et al.*, 2011).

H. influenzae LOS has been proven to induce significant interleukin 6 (IL6), CXCL8, tumour necrosis factor alpha (TNFα) and intercellular adhesion molecule 1 (ICAM-1) production in human bronchial epithelial cells (Khair *et al.*, 1994). There is no research about the inflammatory response caused by *H. parainfluenzae* LOS yet. Those differences in LOS structure suggest that the two *Haemophilus* may play pathogenic roles in different ways.

1.10.2 *H. parainfluenzae* polysaccharide capsule

Although the capsule has not yet been very well described in *H. parainfluenzae*, it is one of the most important virulence factors in *H. influenzae*. To date, six different capsular serotypes have been described for *H. influenzae* (a to f) (Satola, Schirmer and Farley, 2003). Recently, a study described for the first time a capsular operon in *H. parainfluenzae*, a major determinant of pathogenicity that may contribute to increased virulence in extensively drug-resistant (XDR) clinical isolates (González-Díaz *et al.*, 2019). Polysaccharide capsules contribute to bacterial resistance to antimicrobial peptides, enhance bacterial adherence to the cell surface, and also facilitate bacterial biofilm formation.

1.11 How *H. parainfluenzae* colonises in the respiratory tract

The first interaction between bacteria and epithelial cells happens through mucus. The *Haemophilus* species has a high affinity with mucus *in vitro*. The affinity of *H. influenzae* for mucus and the fact they are not efficient at adhering to healthy epithelium may explain why they do not infect healthy airways (Read *et al.*, 1992). In abnormal airways, such as in bronchiectasis airways (Currie, Pavia and Lopez-Vidriero, 1987), mucociliary clearance is delayed and bacteria that have already adhered to the mucus will have more time to establish infection and cause further damage. Leucocytes will be attracted to the airways because of the infection and then generate DNA which makes secretions more viscous and difficult to clear (Currie, Pavia and Lopez-Vidriero, 1987). Sputum of bronchiectasis patients is poorly transported by cilia compared to healthy mucus, which will further accelerate this process.

Piliated bacteria can be more easily trapped by cilia, and the fact most *H. parainfluenzae* don not have cilia helps them penetrate epithelium (Middleton, Chadwick, *et al.*, 2003). *H. parainfluenzae* and *H. influenzae*, as previously mentioned, can slow down the ciliary beating frequency. This contributes to slower mucus clearance. Cilia need to beat in the same direction and coordinate to push out mucus efficiently. In the bacteria-infected area, cilia beat direction is uncoordinated (Bustamante-Marin and Ostrowski, 2017). This contributes to bacterial infection.

As previously described, *H. parainfluenzae* and *H. influenzae* can adhere to bronchial epithelial cells similarly. Several adhesins are known to be present in *H. influenzae*, but not much has been investigated in *H. parainfluenzae*. Pili mediate adherence to all cell types in respiratory cells; it is important for the early stage of colonisation. Most *H. parainfluenzae* contain pili; however, like NTHi, not all of them do (Kahn and Gromkova, 1981). More commonly, *H. parainfluenzae* produces mannose resistant hemagglutinin that can agglutinate animal erythrocytes. *H. parainfluenzae* contains outer membrane protein adhesins which mediate binding to oral streptococci, salivary pellicle, and neuraminidase-treated erythrocytes. *H. parainfluenzae* LOS plays a role in adherence (Liljemark, Bloomquist and Lai, 1991).

H. parainfluenzae and *H. influenzae* can form a biofilm that helps resistance against opsonophagocytic killing by neutrophils. *H. influenzae* can also be found between epithelial cells and inside macrophage-like cells in chronic bronchitis patients (Pickering *et al.*, 2016). More recent studies show that *H. influenzae* can persist intracellularly in the respiratory tract (Clementi and Murphy, 2011; Murphy *et al.*, 2017). This could help *H. influenzae* avoid antibiotics and may contribute to recurrent infections.

1.12 How *H. parainfluenzae* can cause inflammatory responses and damage in bronchiectasis

H. parainfluenzae LOS interacts with toll-like receptor 4 (TLR4) and activates transforming growth factor beta associated kinase-1 (TAK1) through myeloid differentiation primary response 88 (MyD88) pathway, leading to p38 mitogen-activated protein kinase phosphorylation and nuclear factor-κB (NF-κB) activation, which positively regulates transcription of pro-inflammatory cytokines like CXCL8 (Miller, Ernst and Bader, 2005) (Figure 5). CXCL8 is a chemoattractant, which attracts neutrophils to the airways; neutrophils can release proteinase such as elastase to kill bacteria (Miller, Ernst and Bader, 2005). In bronchiectasis, there is already a high level of neutrophil and elastase persistence; elastase can damage the epithelium and stimulate mucus production; this can support bacterial infection by impairing mucociliary clearance. Thus, a vicious cycle may develop.

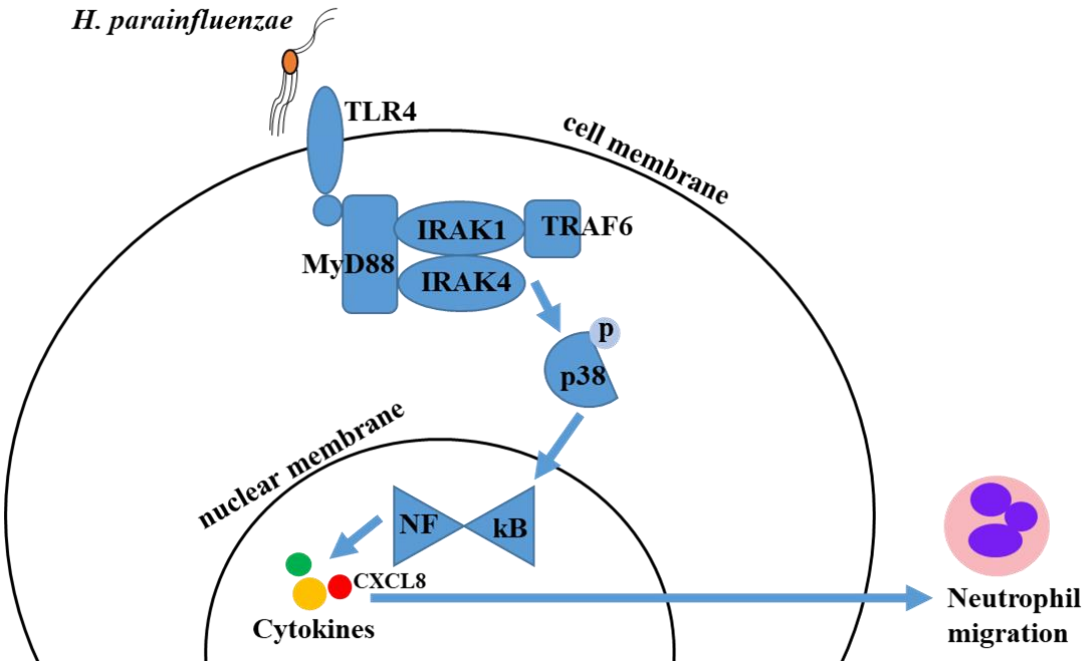


Figure 5. The simplified signal pathway stimulated by *H. parainfluenzae*. *H. parainfluenzae* interact with TLR4, then actives P38 and NFκB through MyD88 pathway. This leads to the production of pro-inflammatory cytokines such as CXCL8 which can attract neutrophil migration.

1.13 Current treatment for *H. parainfluenzae* in bronchiectasis

Although the pathogenesis of *H. parainfluenzae* is under debate, in bronchiectasis, *H. parainfluenzae* are still rarely reported. According to the protocol in Royal Infirmary's bronchiectasis clinic in Edinburgh, *H. parainfluenzae* are considered commensal bacteria, which means patients' sputum samples with *H. parainfluenzae* would be reported as containing mixed normal flora. Therefore, no *H. parainfluenzae* specific antibiotic sensitivity test would be done.

The technique used to process the patient's sputum sample is key for an accurate diagnosis, especially for *Haemophilus* species, which is nutrient fastidious. In the Royal Infirmary of Edinburgh microbiology laboratory, sputum processing is as follows. The sample is hydrolysed using an equal volume of Sputolysin (Calbiochem), then a loop (around 10 µL) of the hydrolysed sample will be diluted with 5 mL of saline. 100 µL of the diluted sample will be plated on blood agar (BA), and chocolate blood agar (CBA), and results read after 24 hours. Using this method, because of the small sample size used for culture (a loop of the sample) and lack of serial dilution, there is a high chance of under-identifying bacteria. There is also no routine quantification carried out. This may underestimate the number of *Haemophilus* species identified.

1.14 Current research about *H. parainfluenzae* in bronchiectasis

As previously mentioned, there has been limited research about *H. parainfluenzae* in airway infection and the debate about whether *H. parainfluenzae* are pathogen or not has remained. With modern techniques, more studies point to *H. parainfluenzae* playing a pathogenic role in different diseases, and more researchers are working on their pathogenicity. The high multidrug resistance associated to *H. parainfluenzae* is also attracting more attention to this area. There is a need to investigate the prevalence of *H. parainfluenzae* in bronchiectasis and, more importantly, whether it has a pathogenic role.

To look into the potential pathogenicity of *H. parainfluenzae*, it is also important to investigate its role in the lung microbiome, and whether this pathogenicity may be influenced by the interaction with other bacteria in bronchiectasis. In Edinburgh Royal

Infirmary bronchiectasis clinic, patients sometimes are found to have more than one dominant pathogen present in their lower airway, but few of them have *Haemophilus* species and *P. aeruginosa* at the same time, even though *Haemophilus* species and *P. aeruginosa* are the two most common pathogens in bronchiectasis. *H. influenzae* and *P. aeruginosa* had been found together in CF patients; however, there is also a tendency to lower numbers of *H. influenzae* in patients chronically colonised by *P. aeruginosa*, and later in the CF, *H. influenzae* may be replaced by *P. aeruginosa* (Riley and Hoffman, 1986). There is limited data about *H. parainfluenzae* as a co-pathogen in bronchiectasis, but there is research suggesting that *H. parainfluenzae* can promote the growth of two laboratory strains of *P. aeruginosa* (Liu A *et al*, 2017). It is still unclear what interaction there is between *H. parainfluenzae* and *P. aeruginosa* or how it could influence the virulence of *H. parainfluenzae* and affect the lung microbiome in bronchiectasis. In this study we looked into the interaction of *H. parainfluenzae* and *P. aeruginosa* as a stepping stone in the understanding of how bacterial interaction contributes to the change of the lung microbiome and, eventually, the host inflammatory responses in bronchiectasis.

1.15 The lung microbiome: what is the microbiome in the lung and how is it associated with disease

The human microbiome is the collection of microorganisms that live in and on the human body. Healthy lower airways have long been considered sterile. This concept has been challenged by robust studies focusing on airway microbiomes. The first study reporting the existence of significant microbiota in the lower airways was published in 2010 (Hilty *et al.*, 2010); after that, more and more studies proved the presence of microbiota in the lower airways. This study identified 16s sRNA bacterial sequences from 8 healthy subjects, as well as 11 asthma patients and 5 COPD patients. They found those three groups have similar kinds of bacteria in their nose, oropharynx and left upper lung. However, there is a significant difference in bacterial community composition in the three patients' groups. This study suggests that not only is there a microbiome in the lung, but that it differs in healthy and diseased lungs.

After that, more studies started to look at the composition of the microbiome in different diseases. The ability to have a diverse microbiome is often impaired in the

diseased lung, which contributes to a heavier bacterial burden. However, this process is not simply an enrichment of all bacteria community, but more likely a selection (Salami and Marsland, 2015). In COPD, pathogenic *Proteobacteria*, particularly the *Haemophilus* species, were much more frequently isolated compared to in healthy controls (Dickson, Martinez and Huffnagle, 2014). In children with asthma, there was also a highly significant increase in *Haemophilus* species, compared to healthy controls (Simpson, Daly, *et al.*, 2016). In idiopathic pulmonary fibrosis, *Haemophilus* and *Streptococcus* were found to be more abundant than in healthy controls (Molyneaux *et al.*, 2014).

Change in the microbiome is also related to the severity of a disease. There is an increased bacterial burden and decreased bacterial diversity in exacerbated COPD compared to stable COPD (Dickson, Martinez and Huffnagle, 2014). In adult neutrophilic asthma compared to non-neutrophilic asthma, there is significantly more *Proteobacteria,* and interestingly, significantly fewer *Actinobacteria* and *Firmicutes*. The colonisation of *Haemophilus* species was significantly associated with increased risk of viral infection in the lower airways (Simpson, Daly, *et al.*, 2016). In bronchiectasis, patients who have *P. aeruginosa* or *H. influenzae* dominated communities have significantly higher serum CRP, sputum CXCL8 and interleukin 1 beta (IL1β), worse lung function and more frequent exacerbations. These changes usually lead to a reduced richness and diversity of bacterial species in the community, as well as a shift in microbiome composition. In a case of early childhood asthma, the colonisation started from *Staphylococcus* and *Corynebacterium*; after one year, these had been replaced by *Alloicoccus* and *Moraxella* (Simpson, Daly, *et al.*, 2016). The microbiome often shifts from healthy lung dominated bacteria species to a phylum that contains pathogenic bacteria such as *Proteobacteria.* It is still under debate, however, whether this shift is driven by antibiotic usage or disease development.

Current knowledge about the microbiome in the lung does not clarify whether it is an altered lung microbiome that leads to disease development or viceversa. An altered airways' microbiome should also be considered on top of the traditional single pathogen model, therapies targeting at changing microbiome may be a future solution to prevent disease progression.

1.16 The microbiome is the community

To understand the microbiome, change in lung diseases, it is important to understand how the dominant pathogen wipes out the others. With this purpose, we should start to look at bacteria as social creatures in a community called the microbiome. Bacteria can communicate within their species or interspecies, and they can form 'group activities' (Stacy *et al.*, 2016). The interactions between bacteria impact the microbiome composition, their biological activities and finally, change disease severity. For example, in a wound model, the interaction between *P. aeruginosa- S. aureus* leads to a more severe infection, and a higher resistance to antibiotic treatment compared to *P. aeruginosa* infections on their own (DeLeon *et al.*, 2014). Oppositely, *Bifidobacteria* can inhibit the translocation of *Escherichia coli* (*E. coli*) Shiga toxin from the gut lumen to the blood through production of acetate, thereby protecting the host from *E.coli* infection (Fukuda *et al.*, 2011). Bacterial interaction can be synergistic or antagonistic; this mainly depends on how bacteria communicate.

1.17 How do bacteria communicate?

Bacteria interact in many ways. Their interaction can be parasitic, mutualistic or commensal bacteria. They interact directly or indirectly (Waters and Bassler, 2005)(Dworkin, 1999).

Direct interaction, also called physical interaction, is commonly used and requires actual cell-to-cell contact. Bacteria establish a multi-bacterial structure by getting close to each other; this can contribute to further indirect interaction between cells or facilitate bacterial invasion to cells. Physical interactions are often mediated by pili. Bacteria usually exchange information (such as chemicals, proteins or genetic materials) through it from one bacteria to another (Dworkin, 1999). It can also be achieved by bacteria recognising each other through receptors and adhesins on the surface of bacteria (Jakubovics *et al.*, 2006). These two processes all require a short distance between bacteria for physical contact.

Indirect or chemical interaction facilitates bacterial information exchange through long distances using chemical molecules. Bacteria produce and release chemical molecules to the environment, the chemical signal accumulates and the external concentration

increases, the other bacteria can detect the signal when the concentration reaches the minimum threshold, then it can stimulate changes in bacterial gene expressions, and bacterial behaviour. This process is called quorum sensing (Waters and Bassler, 2005).

There are four most common examples of bacterial interactions: co-aggregation (physical), biofilm remodelling (physical), local growth inhibition (chemical) and local growth promotion (chemical) (Stacy *et al.*, 2016).

Co-aggregation is a multi-species bacterial mixture which is mediated by recognition of specific surface molecules. Co-aggregation is a very common phenomenon for bacteria isolated from the dental plaque (Ganpshkumar, 2017), although it has also been found in the gut and urogenital tract (Reid *et al.*, 1990). In co-aggregation, bacteria are attached to a specific group of bacteria. It is unusual for one bacterium to bind to a wide variety of bacteria. Bacteria that do not have competitive adhesins and receptors can use other bacteria as a bridge to co-aggregate. Sometimes they can develop into a co-aggregation net (Jakubovics *et al.*, 2006).

Biofilm remodelling. A biofilm is a dynamic structure; it allows bacteria to reach an optimum position to get nutrients and oxygen. For example, when culturing *P. aeruginosa* and *S. aureus* together, *P. aeruginosa* can form a cap-like biofilm on top of *S. aureus. P. aeruginosa* dominates the upper layer while *S. aureus* is mainly persistent at the bottom. In this way, *P. aeruginosa* can obtain more nutrients and oxygen; at the same time, if the *P. aeruginosa* is an ampicillin resistant strain, this cap protects *S. aureus* from the antibiotic. (Kim *et al.*, 2014) Polysaccharide synthesis locus (Psl), a major polysaccharide in *P. aeruginosa*, which is required in this process, is strongly selected in the lungs of patients with cystic fibrosis (Jackson *et al.*, 2004; Hogardt and Heesemann, 2010).

Local growth promotion. Bacterial growth can be promoted by receiving beneficial metabolites from distant bacteria. This bacterial communication is called metabolic cross-feeding (Ramsey, Rumbaugh and Whiteley, 2011).

Production of a beneficial metabolite is an important bacterial communication tool. Bacteria can consume the by-product of another by metabolic cross-feeding. *Streptococcus gordonii* (*S. gordonii)* produces L-lactate and H_2O_2. The production of

L-lactate provides *Aggregatibacter actinomycetemcomitans* with a preferred carbon source for growth and reduces the need to compete with *S. gordonii* for glucose during aerobic co-culture (Ramsey, Rumbaugh and Whiteley, 2011). This metabolic synergy results in a mutualistic relationship between both species during an abscess co-infection in which both bacteria reach higher burdens when together rather than when apart.

Local growth inhibition. Metabolic waste products can also act as broad-spectrum toxins, affecting bacterial growth near the producer bacteria. For example, *Bifidobacteria* can inhibit the translocation of *E. coli* Shiga toxin from the gut lumen to the blood through the production of acetate, thereby protecting the host from *E.coli* infection (Fukuda *et al.*, 2011).

1.18 How the microbiome and bacterial communication influence bronchiectasis

Across many respiratory diseases, it has been shown that disease is associated with a loss of bacterial diversity and richness, or the dominance of a single kind of bacteria or a small group of bacteria, which leads to a loss of evenness in the microbiota. The richness and diversity of bacterial composite show a positive correlation with lung function in bronchiectasis (Woo *et al.*, 2019), although it cannot be stated whether this result is causal or due to frequent antibiotic exposure. *Proteobacteria*, which include *Pseudomonas* and *Haemophilus*, usually dominate the diseased airway in bronchiectasis and are associated with higher neutrophil-mediated inflammation and exacerbations.

Interactions between species could influence their virulence and pathogenicity, contribute to changes in the microbiome, and eventually influence the severity of the disease. There are a few different ways in which the microbiome or bacterial interactions can contribute to airway damage: interspecies interaction may encourage the production and release of virulent traits or metabolic interactions may change bacterial growth and alter the environment. Genetic material exchange may develop higher antibiotic resistance, and mixed biofilm formation increase microbiota resilience (B.Rogers, 2018) (Figure 6).

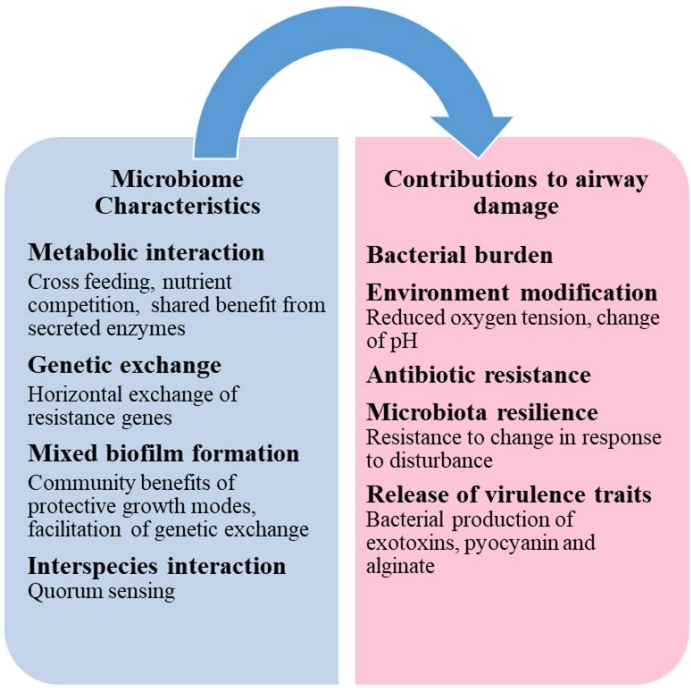


Figure 6. The influence from the microbiome to bronchiectasis (B.Rogers, 2018)

There is still much to learn about the changes in the microbiota that may be relevant to the development of bronchiectasis and its progression. There is also much to know about bacterial interactions in the airways and bronchiectasis.

Most of the research on bacterial communication has used laboratory strains of bacteria, and mainly focused on acute infections. Research on bacterial interactions in bronchiectasis will help understand the microbiome found in chronic lung disease and may give further insight on the strategies needed to treat bacterial infection in bronchiectasis.

1.20 Hypothesis

1.20.1 *H. parainfluenzae* are common in bronchiectasis, and in high bacterial loads, it can play a similar pathogenic role as NTHi.

- *H. parainfluenzae* can be isolated from lower airways in bronchiectasis patients.
- *H. parainfluenzae* isolates from lower airways and upper airways may be gnomically different.
- The clinical symptoms found in bronchiectasis patients with *H. parainfluenzae* and NTHi are similar.
- *H. parainfluenzae* can stimulate an inflammatory response from epithelial cells.

1.20.2 *H. parainfluenzae* can induce a stronger inflammatory response when combined with *P. aeruginosa*.

1.21 Aims

- Classify the *Haemophilus* species found in stable bronchiectasis patients.
- Compare *H. parainfluenzae* isolates from bronchiectasis patients' upper airways and lower airways.
- Compare clinical features of *H. parainfluenzae* and NTHi in bronchiectasis patients.
- Assess the impact of *H. parainfluenzae* on epithelial cells.
- Assess the impact of coinfection of *H. parainfluenzae* with *P. aeruginosa* and *H. parainfluenzae* with NTHi.

Chapter 2

Material and Methods

Chapter 2: Material and Methods

The proposed studies only commenced after ethics and R+D approvals. All patients and healthy volunteers filled in a consent form before participating.

2.1 Study designs

2.1.1 Classify the *Haemophilus* species found in bronchiectasis patients

To understand *Haemophilus* species in bronchiectasis, a total of 140 stable patients were recruited. Patients' sputum samples were collected and processed for microbial culture. Multiple techniques (described later in this chapter) were performed to classify the *Haemophilus* species.

Sputum samples from another 51 unselected patients were also sent to NHS microbiology laboratory to compare the outcomes from our proposed method.

2.1.2 Compare *H. parainfluenzae* isolates from bronchiectasis patients' upper and lower airways

From 30 stable bronchiectasis patients recruited, sputum and throat swab samples were collected and processed for microbial culture. *Haemophilus* species were isolated from the samples and stored at -80^{o}C. Their genomic profile was analysed.

The inflammatory effect of *H. parainfluenzae* cultured from same patient's upper and lower airways was tested on human bronchial epithelial cell line (16HBE). Cell cytokine production CXCL8 was measured.

2.1.3 Compare clinical features of *H. parainfluenzae* and NTHi in bronchiectasis patients

Blood and sputum samples were collected from 63 stable bronchiectasis patients that had either NTHi or *H. parainfluenzae* isolated from their sputum culture. Their clinical features, sputum and serum inflammatory markers were compared. Serum White cell counts (WCC), ESR, CRP and ICAM-1 were measured as well as sputum sol phase myeloperoxidase (MPO).

2.1.4. Assess the impact of *H. parainfluenzae* on epithelial cells

In order to understand the pathogenicity of *H. parainfluenzae,* the inflammatory effect of *H. parainfluenzae,* NTHi and *P. aeruginosa* on 16HBE cells, human primary bronchial epithelial cells (NHBE) and bronchiectasis patient primary nasal epithelial cells was tested. Cytokine production was measured using multiple methods.

2.1.5 Assess the impact of coinfection of *H. parainfluenzae* with *P. aeruginosa* and *H. parainfluenzae* with NTHi

To understand the interaction between bacteria and the changes to the microbiome in bronchiectasis, 16HBE cells were co-infected by *H. parainfluenzae* and *P. aeruginosa*, the two most common pathogens in bronchiectasis. To further unveil the mechanics, 16HBE cells were challenged by *H. parainfluenzae* in multiple conditions: with broth that had previously cultured *P. aeruginosa*, with cell media that had previously cultured *P. aeruginosa* and with cell media that had co-cultured cells and *P. aeruginosa*. Cellular inflammatory response and bacterial loads were measured after cellular co-culture.

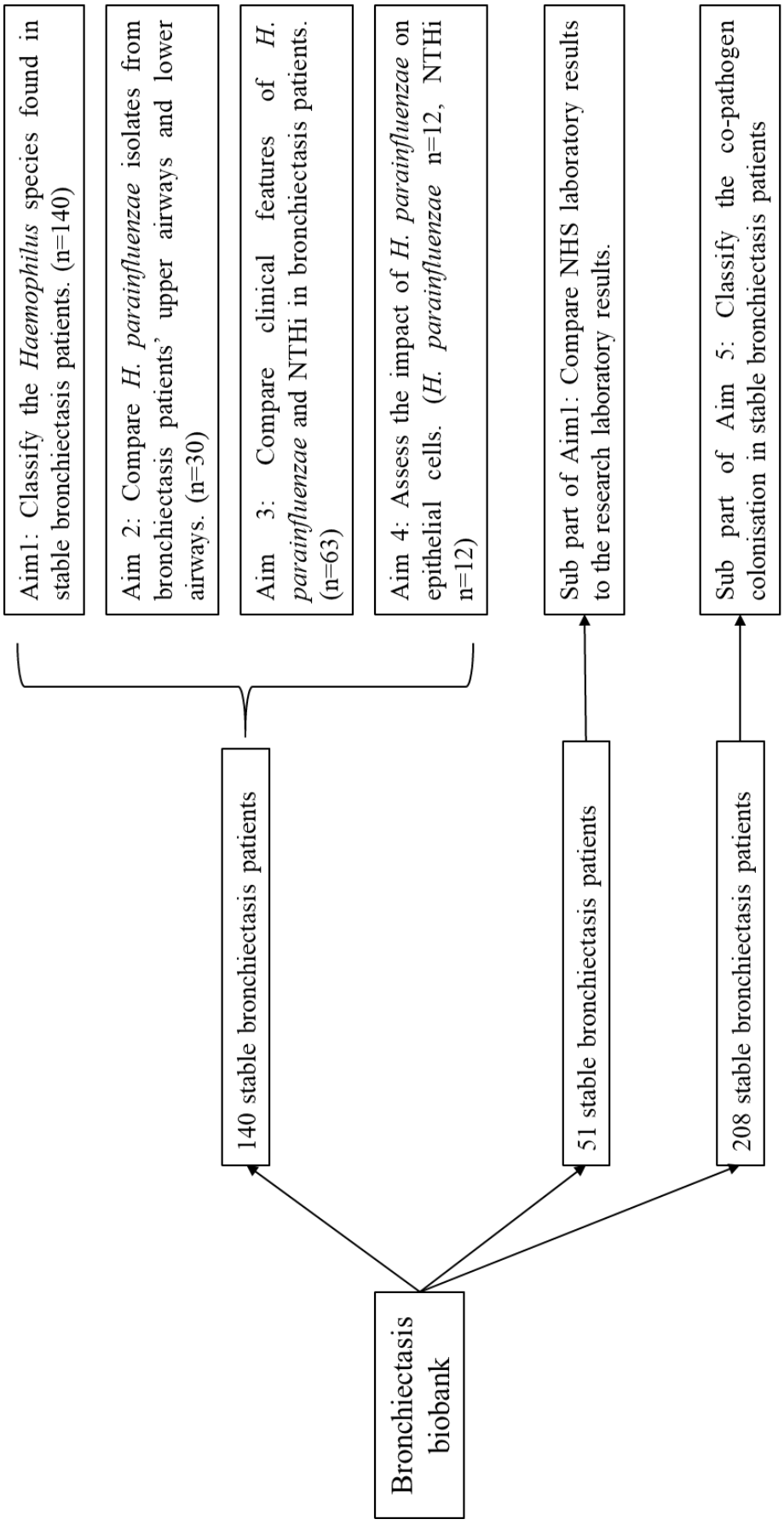


Figure 7. Patient groups for aim 1 to aim 5

2.2 Study Methods

2.2.1 Classify *Haemophilus* species in bronchiectasis patients

2.2.1.1 Patient recruitment and sample collection

A. Inclusion criteria of bronchiectasis patients

Patients were chosen from the bronchiectasis clinic in the Royal Infirmary of Edinburgh. Inclusion criteria for stable patients included: patients with clinically significant bronchiectasis; 18 years old and older; no clinical exacerbations for at least 4 weeks; not on long term oral or inhaled antibiotics; *H. influenzae* found in the last sputum culture within the past year according to NHS microbiology reports.

B. Patient information

Information on patient's age, sex, smoking status, body-mass index (BMI), spirometry, bronchiectasis severity index and medical history were collected.

Bronchiectasis severity index (BSI): BSI uses specific criteria (Table 1) and is calculated with the assistance of an online calculation tool (http://www.bronchiectasisseverity.com/15-2/). BSI scores of 1-4 are considered mild bronchiectasis, 5-8 are moderate bronchiectasis, and 9 or more is considered severe bronchiectasis.

Table 1. Bronchiectasis severity index

Variable	BSI
Age	
50-69	2
70-79	4
80+	6
BMI <18.5 kg/m^2	2
≥3 lobes involved (or cystic bronchiectasis)	1
FEV_1 % Predicted	
50-80	1
30-49	2
<30	3
Hospital admission in the last 2 years	5
Exacerbations in the last year ≥3	2
Dyspnoea MRC score	
4	2
5	3
Pseudomonas colonization	3
Colonization with other organisms	1

C. Patients sample collection

Spontaneous sputum was collected from patients.

2.2.1.2 Sputum processing

The proposed method was based on our laboratory methodology (Figure 7). The standard methods used in the NHS microbiology laboratories is shown for comparison.

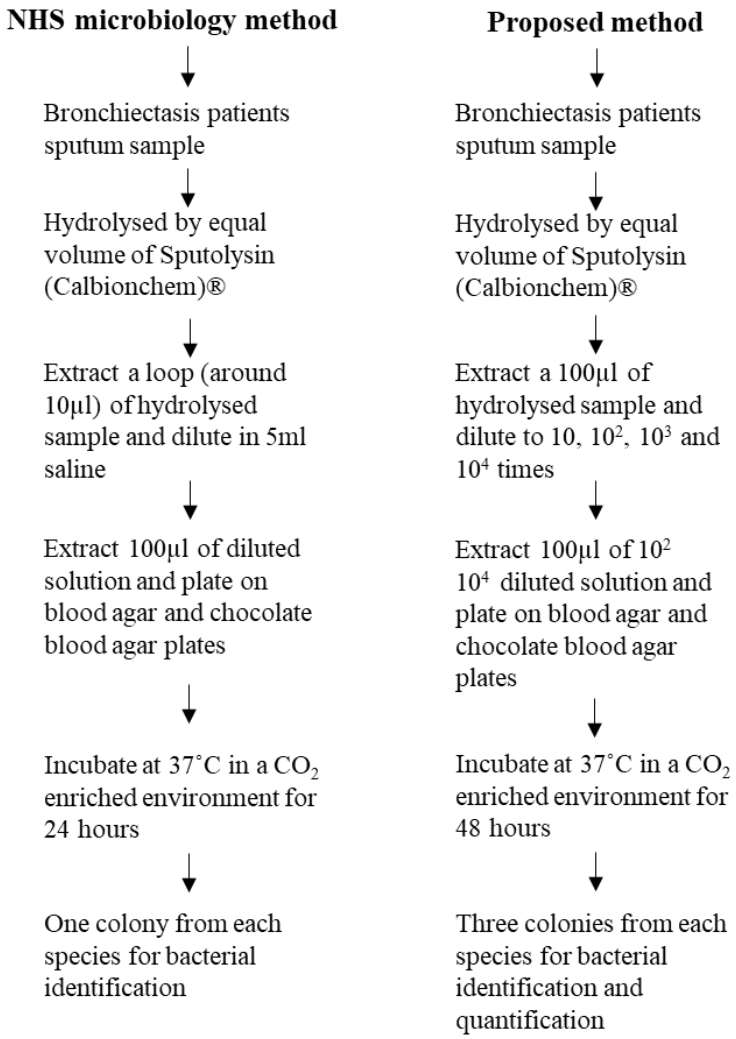


Figure 8. Sputum processing protocol. Methods from NHS microbiology laboratory method and our proposed method

Materials and equipment

- Sputolysin (Calbiochem®)
- 0.85% sterile saline
- BA (Columbia blood agar base + 5% defibrinated horse blood – Oxoid®)
- CBA (heated Columbia blood agar base + 5% defibrinated horse blood – Oxoid®)
- Pseudomonas isolation agar (PIA)(Difco®)
- Sterile tubes
- 37 ˚C incubator
- 37 ˚C carbon dioxide incubator

- Ultracentrifuge maximum speed up to 50,000×g (Sorvall Discovery 100AC)
- -80 ˚C freezer

Methods

In accordance with the proposed method (Figure 6), sputum sample was hydrolysed using Sputolysin and then serially diluted to 1 in 10,000 times with sterile saline. 100 µL of diluted sputum was plated onto BA plate, CBA plate and PIA plate. Plates were cultured in 37 ˚C for 48 hours, CBA plates were incubated in an aerobic atmosphere enriched with 5-10 % carbon dioxide.

After 48 hours of culture, colony numbers on plates were counted. Bacterial load was calculated according to the dilutions. Three colonies were collected for future research.

2.2.1.3 Bacterial identification for *S. aureus, S. pneumoniae, P. aeruginosa* and other Gram-negative bacteria

After the growth of bacteria, different techniques were used for bacterial identification.

Materials and equipment

- Staphaurex Plus kit (Bronidox®)
- Optochin discs (Mast Group Ltd®)
- API 20E and API 20NE kits ((bioMerieux UK Limited, Basingstoke, Hampshire)
- BA (Columbia blood agar base + 5% defibrinated horse blood – Oxoid®)
- CBA (heated Columbia blood agar base + 5% defibrinated horse blood – Oxoid®)
- Pseudomonas isolation agar (PIA)(Difco®)
- MacConkey agar

Methods

A. *S. aureus*

S. aureus grows on CBA plates, form large, round and yellow colonies. They are Gram-positive cocci, usually found in grape-like (staphylo-) clusters.

Staphaurex Plus kit from Bronidox® was used to confirm *S. aureus*. The reagent contains yellow latex particles, those particles were coated by *S. aureus* specific fibrinogen and rabbit IgG. During the test, mix a drop of this reagent with *S. aureus* organisms, strong agglutination happens in 30 seconds between *S. aureus* clumping factor and fibrinogen, *S. aureus* protein A and the fragment crystallizable of IgG, or *S. aureus* cell surface antigens and specific IgG (Figure 8).

a)

b)

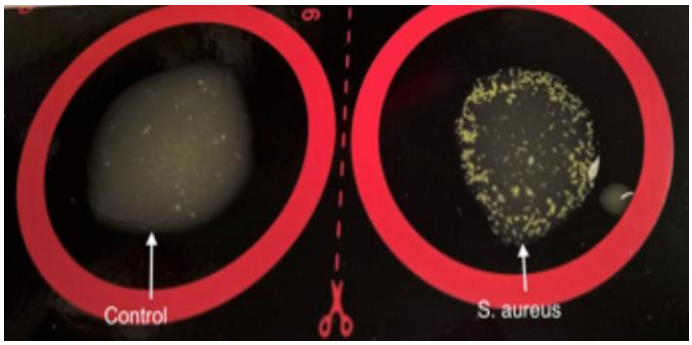


Figure 9. *S. aureus* colony appearance and Staphaurex assay results a) *S. aureus* colonies on blood agar plate. b) Staphaurex assay results for negative control and *S. aureus*. In the positive test, there is clumping of particles formed by *S. aureus* and fibrinogen.

B. ***S. pneumoniae***

S. pneumoniae grow on CBA plates, form small (1-2mm in diameter), round and white colonies. *S. pneumoniae* are alpha-haemolytic. They are Gram-positive cocci, often found in pairs, chains or clusters.

S. pneumoniae were confirmed by optochin text (optochin discs - Mast Group Ltd.). Different from other *streptococci*, *S. pneumoniae* is sensitive to ethylhydrocuprein hydrochloride (optochin). Optochin discs are paper discs that already impregnated with optochin. For the test, one *S. pneumoniae* colony was streaked all over blood agar, then place one optochin disc in the centre of the plate. Incubate the plate at 37 ˚C for 24 hours. *S. pneumoniae* culture shows a zone of inhibition around the impregnated disc (Figure 9).

Figure 10. *S. pneumoniae* test results. A positive result for *S. pneumoniae* test, showing growth inhibition around the optochin disc.

C. *P. aeruginosa*

P. aeruginosa grows on PIA plates, their colony appearance varies. The most common ones are 2-3mm diameter and round shape. Colony colour changes depending on the production of PCN (blue-green), pyoverdine (yellow-green, fluorescent), or pyorubin (red-brown, produced by a small proportion of strains). Some strains do not produce PCN. *P. aeruginosa* have a characteristic smell of aminoacetophenone. There are both mucoid and non-mucoid *P. aeruginosa*. *P. aeruginosa* are gram-negative bacteria.

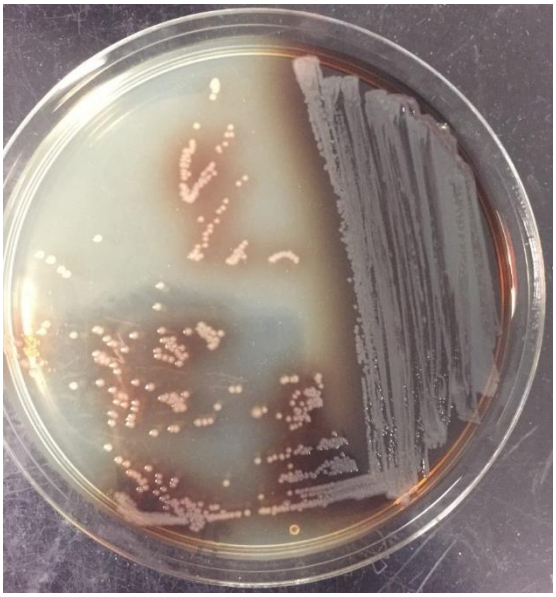


Figure 11. *P. aeruginosa* colony appearance. *P. aeruginosa* colonies on PIA plates. This non-mucoid *P. aeruginosa* shows brown staining on the PIA plate.

D. Other Gram-negative bacteria

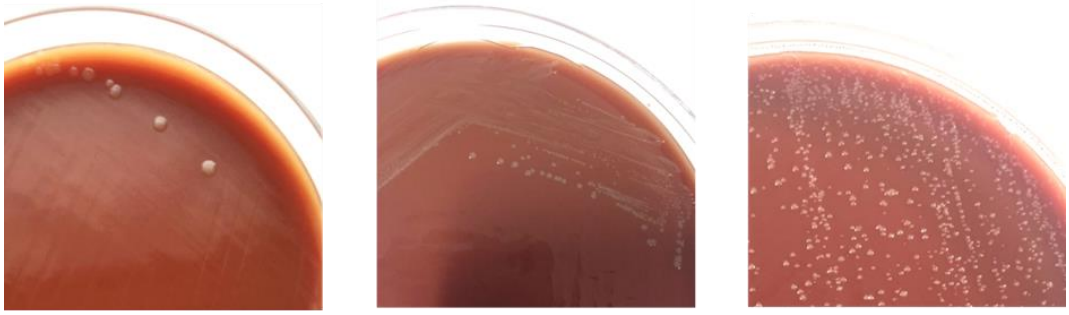
Other Gram-negative bacteria identified using API 20E and API 20NE kits from BIOMERIEUX®. They were plated on MacConkey agar plates, MacConkey agar can prevent the growth of gram-positive bacteria, and differentiate bacteria that can ferment the sugar lactose or not.

Bacteria that cannot ferment the sugar lactose were further tested using API20NE test. According to the manufacturer's directions, around one to four colonies were suspended in 2 mL of 0.85% sterile saline. There are twenty micro-tubes containing dehydrated substances in API 20NE test strip. These micro-tubes were inoculated with the bacterial suspension for 24 hours or 48 hours at 30°C with the addition of reagents and interpretation of reactions carried out according to the manufacturer's directions. The biochemical reactions were converted accordingly into an octal profile number and decoded using the Analytical Profile Index (API Database Vn6.0, APILAB Software Vn3.3.3, Apilab Plus; bioMerieux)

Bacteria that can ferment sugar lactose were further tested using API 20E test according to the instructions, similar to API 20NE test.

2.2.1.4 Bacterial identification for *Haemophilus* species to differentiate NTHi, *H. parainfluenzae* and *H. haemolyticus*

Haemophilus species grow on CBA plates. They are small, grey or transparent round colonies (Figure 11). They are gram-negative coccobacillus.



a) b) c)

Figure 12. *Haemophilus* species. a) NTHi; b) *H. parainfluenzae* and c) *H. haemolyticus* on CBA plates

A. Identify *H. parainfluenzae*

For identification of *H. parainfluenzae*, the XV test was used.

Different from *H. influenzae* and *H. haemolyticus*, the growth of *H. parainfluenzae* only requires V factor rather than both X and V factor. XV test based on this theory is commonly used to differentiate *H. parainfluenzae* from *H. influenzae* (Hinz et al. 2015).

Materials and equipment

- X+V factor discs (Mast Group Ltd.)
- Nutrient agar (Columbia Blood Agar Base, Oxoid®)
- 37 ˚C incubator with 5% CO_2

Methods

One colony was streaked all over the nutrient agar, then placed the paper discs that containing X factor, V factor and X+V factor on the surface of the agar in the configuration of an equilateral triangle with a minimum of 3.5 cm distance between discs. Then incubated at 37 ˚C with 5% CO_2 for 24 hours. *H. parainfluenzae* grew

around both V factor disc and X+V factor disc, while *H. influenzae* and *H. haemolyticus* only grew around X+V factor disc (table 2). This experiment was repeated for three separate colonies.

Table 2 Growth factors for *Haemophilus* species

	X factor (hemin)	V factor (nicotinamide adenine dinucleotide)	X+V factor
H. influenzae	-	-	+
H. haemolyticus	-	-	+
H. parainfluenzae	-	+	+

B. Identify *H. haemolyticus*

qPCR assay was performed to identify *H. haemolyticus*. *H. influenzae* are positive for *Fuculose Kinase* (*fucK*) and *H. influenzae* specific protein D genes (*Hi hpd*), *H. haemolyticus* is positive for [Cu, Zn]-superoxide dismutase C (*sodC*) and *H. haemolyticus* specific *hpd* genes (*Hh hpd*) (Table 3) (Roger Latham, 2015). Figure 12 shows the qPCR result of a clinical strain of *H. influenzae*, a laboratory strain of *H. influenzae* and a laboratory strain of *H. haemolyticus*.

Table 3 Genes used to differentiate *H. influenzae* and *H. haemolyticus*

	Fuculose kinase	*Hi hpd*	*Sod C*	*Hh hpd*
H. influenzae	+	+	-	-
H. haemolyticus	-	-	+	+

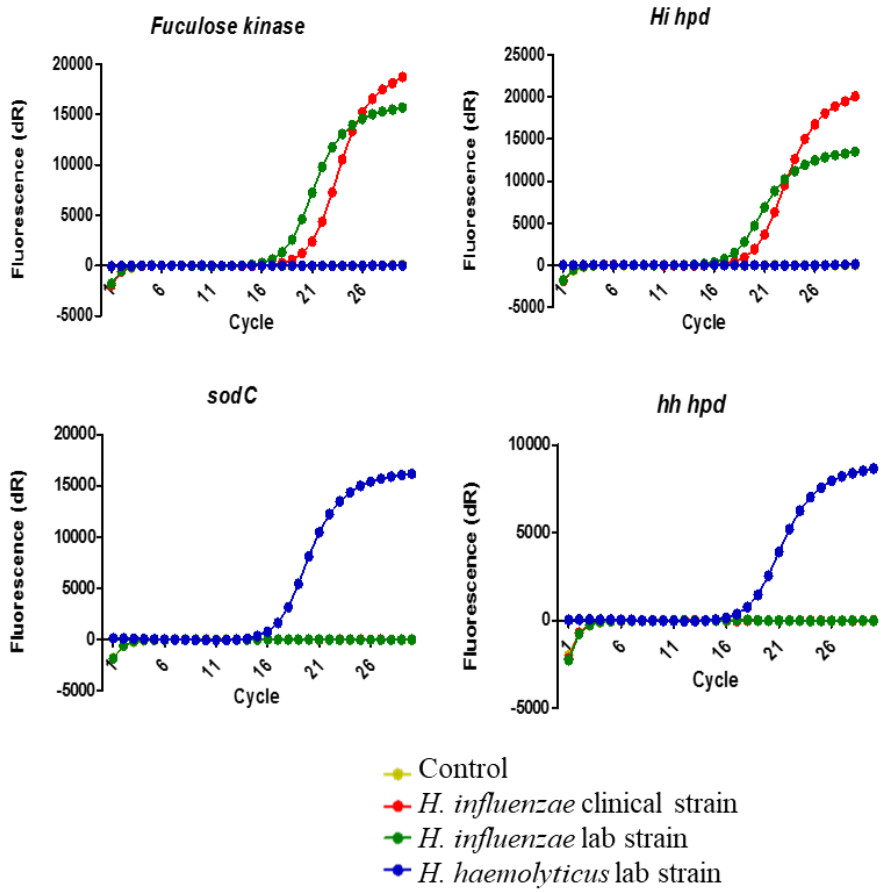


Figure 13. qPCR result for genes *Fuculose kinase, Hi hpd, SodC and Hh hpd*. Red dots stand for the patient strain. Green dots represent a laboratory strain *H. influenzae*; blue dots are a laboratory strain *H. haemolyticus*. The Y axis shows the fluorescence rate of the tested genes. The X axis stands for the qPCR cycle number.

Preparation for qPCR: DNA abstraction:

Bacterial genomic DNA was abstracted using Wizard® Genomic DNA Purification Kit.

Materials and equipment

- Brain heart infusion broth (BHI broth)
- Wizard® Genomic DNA Purification Kit contains: Nuclei Lysis Solution, RNase Solution, Protein Precipitation Solution and DNA Rehydration Solution (Wizard®)
- Isopropanol, room temperature
- 70% ethanol, room temperature
- Water bath, 80 °C
- Water bath, 37 °C
- 1.5 mL micro centrifuge tubes
- Nanodrop spectrophotometer (Thermo Scientific)
- Centrifuge make and model of centrifuge
- *H. haemolyticus* laboratory strain: NCTC10659 (NHS microbiology laboratory collection)
- *H. influenzae* type c laboratory strain: ATCC9007 (NHS microbiology laboratory collection)

Methods

Bacteria were cultured in BHI broth overnight. The next day, 1mL of the overnight culture was collected and centrifuged at 13,000–16,000×g for 2 minutes to pellet the cells. Removed the supernatant and added 600 µL of Nuclei Lysis Solution, incubated in 80 °C for 5 minutes to lyse the cells. After cooled down to room temperature, added 3 µL of RNase Solution to the cell lysate and incubated at 37 °C for 15–60 minutes. Cooled the sample to room temperature. Then added 200 µL of Protein Precipitation Solution to the RNase-treated cell lysate. Vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. Incubated the sample on ice for 5 minutes. Then centrifuged at 13,000–16,000×g for 3 minutes.

Transferred the supernatant containing the DNA to a clean 1.5 mL micro centrifuge tube containing 600 µL of room temperature isopropanol, and gently mixed by inversion until the thread-like strands of DNA form a visible mass. Then centrifuged at 13,000–16,000×g for 2 minutes. Carefully poured off the supernatant and drained the tube on clean absorbent paper. Added 600 µL of room temperature 70% ethanol and gently inverted the tube several times to wash the DNA pellet. Centrifuged at 13,000–16,000×g for 2 minutes again and carefully aspirated the ethanol. Drained the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes. After the pellet dried added 100 µL of DNA Rehydration Solution to the tube and rehydrated the DNA by incubating at 65 °C for 1 hour. Periodically mix the solution by gently tapping the tube. Final DNA product was stored at 2–8 °C.

Copy number of DNA product was performed using Nanodrop spectrophotometer.

qPCR set up

Material and equipment

- SensiFAST SYBR (Bioline, BIO-98005)
- Primer (Eurofins genomics)
- Distilled water
- 0.1 mL tubes
- Thermal cycler (Rotor-Gene Q, Qiagen)

Methods

Primers were referenced from Latham's study (Latham, Zhang, & Tristram) (Table 4). The reaction volumes for the qPCR assays were 15 µL in 0.1 mL tubes and contained 100 pg of template, 333 nM each primer, and 7.5 µL SensiFAST SYBR. Thermo cycling consisted of an initial melt at 95 °C for 3 minutes followed by 30 cycles of 95 °C for 10 seconds, 60 °C for 10 seconds and 72 °C for 10 seconds. Data were acquired at 72 °C, and a melt curve was collected between 60 °C and 95 °C. All qPCR amplification delivered a single melt peak with cycle threshold between 13 and 18.

Table 4. Primers information for qPCR

Target	Primer	Primer sequence (5' to 3')	Expected amplicon size (bp)
H. haemolyticus sodC	sodC_F	GTGCGGTATGTTCAGTTG	163
	sodC_R	AGGCCATAGTTAGATTCAGTAAT	
H. influenzae fucK	fucK_F	TCAATGCTCACTSAACGCTTAAC	150
	fucK_R	ACGCATAGGAGGGAAATGGT	
H. haemolyticus Hh-hpd	Hh-hpd_F	GCGAAAGGTMAATGGGTAAACTAC	131
	Hh-Hhpd_R	TAATGGTGTGTAKACAATGTT	
H. influenzae Hi-hpd	Hi-hpd_F	AAGGGTTATTGGGTAAACTATA	156
	Hi-hpd_R	TCAGGTTTGGATTCTTCTTTATTA	

C. Identify NTHi and type-able *H. influenzae*

PCR technique was used to identify NTHi. Capsular export gene *bexA* and *bexB* were found exclusively on the cap locus of the bacteria, which encodes the polysaccharide capsule, and therefore were not present in NTHi strains. Gene *pepN*, which is necessary for the glutathione metabolism of all *H. influenzae*, was chosen as a positive control (Table 5) (Davis, G. S. *et al.* 2011). Figure 13 shows the results for 2 clinical strains of NTHi (P1 and P2).

Table 5. Genes used to differentiate NTHi and Capsule *H. influenzae*

	bexB	*bexA*	*pepN*
Capsule *H. influenzae*	+	+	+
NTHi	-	-	+

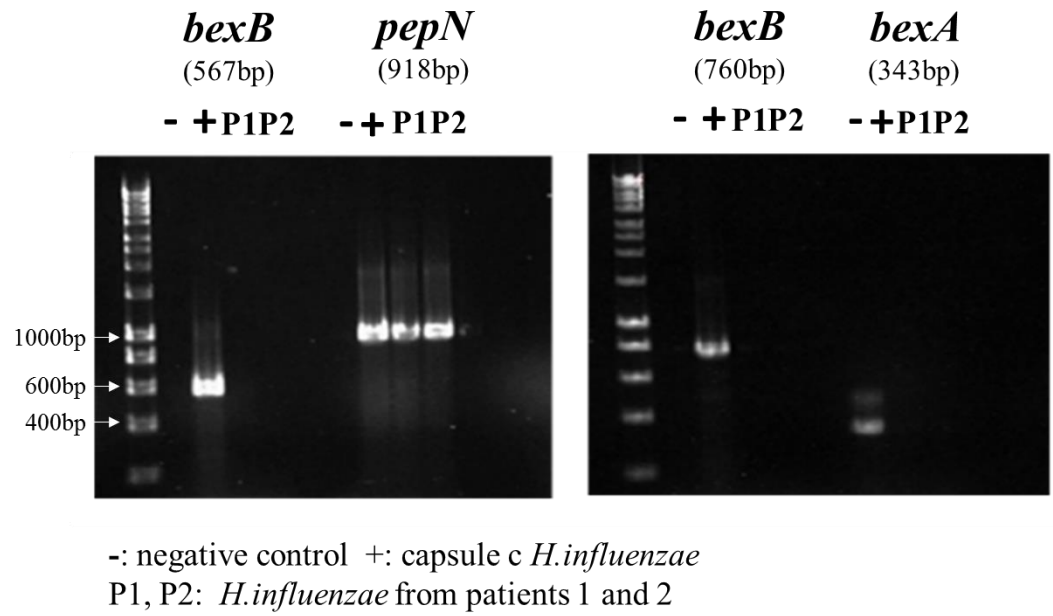


Figure 14. Electrophoresis result of *H. influenzae* strains from patients P1 and P2. Two pairs of primers for *bexB* gene were used. Previously described *bexA*-specific primers produce a 343-bp amplicon, *bexB* primers used in this study amplify 567-bp and 760-bp products, *pepN* primers amplify 918-bp products. – stands for negative control. + is strain ATCC9007, which is type c capsule *H. influenzae*. P1 and P2 are the NTHi colonies isolated from patient sputum samples.

Preparation for PCR: DNA cell lysate

Materials and equipment

- Distilled water
- 95 °C heat block incubator
- Ice
- Centrifuge
- -20 °C freezer
- *H. influenzae* type c laboratory strain: ATCC9007 (NHS microbiology laboratory collection)

Methods

Two to three colonies were picked up from chocolate blood agar and suspended in 100µL sterile distilled water. The suspension was heated at 95 °C for 10 minutes, then chilled on ice immediately for 5 minutes. The tubes were then centrifuged at 12,000×g for 3 minutes and the supernatant stored at -20 °C for PCR. (Davis, G. S. *et al.* 2011)

PCR set up

Materials and equipment

- Mytag reaction buffer (Bioline®)
- Primers (Eurofins genomics®)
- tagDNA polymerase (Bioline®)
- Distilled water
- Agarose (Sigma®)
- TBE buffer
- 0.1 mL tubes
- Thermal cycler

Methods

Primers of *bexA*, *bexB* and *pepN* were referenced from Davis et al, and two pairs of *bexB* primers were used (Table 6). Capsulated *H. influenzae* ATCC9007 from NHS laboratory is a capsulated type C *H. influenzae* was used as the positive control.

For each reaction, 5 μL Mytag reaction buffer, 2 μL template (the cell lysate made from the last step), 1.5 μL primers, 0.25 μL tagDNA polymerase and distilled water made up to in total to 25 μL in 0.1 mL tubes. Thermo cycling consisted of an initial denaturation at 95 ℃ for 1 minute, then followed by 30 cycles of 95 ℃ for 15 seconds, 55 ℃ for 15 s and 72 ℃ for 10 seconds. The final extension was 72 ℃ for 2 minutes. PCR products were confirmed by running on 10% agarose gel.

Table 6. Primer information for PCR

Target	Primer	Primer sequence (5' to 3')	Expected amplicon size (bp)
bexB	bexB.1F	GGTGATTAACGCGTTGCTTATGCG	567
	bexB.1R	TTGTGCCTGTGCTGGAAGGTTATG	
	bexB.FLF	TCATTGTGGCTCAACTCCTTTACT	760
	bexB.FLR	AGCTATTCAAGGACGGGTGATTAACGC	
bexA	HI-1	CGTTTGTATGATGTTGATCCAGAC	343
	HI-2	TGTCCATGTCTTCAAAATGATG	
pepN	pepN_F	GATGGTCGCCATTGGGTGG	918
	pepN_R	GATCTGCGGTTGGCGGTGTGG	

D. Identify *H. parainfluenzae* subgroups

H. parainfluenzae were bio typed by their production of indole, urease and ornithine decarboxylase (Table 7).

Materials and equipment

- API NH kits (bioMerieux UK Limited, Basingstoke, Hampshire)
- Incubator

Methods

The production of indole, urease and ornithine decarboxylase were tested using API NH kits (bioMerieux UK Limited, Basingstoke, Hampshire) according to the instructions, similar to the previously described API20NE test.

Table 7. Chemicals produced from *H. parainfluenzae* subtype I to VIII

Biotype	*Indole*	*Urease*	*Ornithine decarboxylase*
I	-	-	+
II	-	+	+
III	-	+	-
IV	+	+	+
V	-	-	-
VI	+	-	+
VII	+	+	-
VIII	+	-	-

2.2.1.5 Bacteria storage

To preserve bacteria, bacteria were stored in a freezer at -80 °C in 1mL 10% skimmed milk (Oxoid®).

2.2.2 Compare *H. parainfluenzae* isolates from bronchiectasis patients' upper airways and lower airways

2.2.2.1 Inclusion criterial of bronchiectasis patients

Thirty patients were chosen from the bronchiectasis clinic in the Royal Infirmary of Edinburgh. Inclusion criteria for the stable patients included: patients with clinically significant bronchiectasis; 18 years old and older; have not had an exacerbation for at least 4 weeks; were not on long term oral or inhaled antibiotics; have grown *H. influenzae* in the last sputum culture within the past one year according to NHS microbiology reports.

2.2.2.2 Patients sample collection

Spontaneous sputum and throat swabs were collected from patients.

2.2.2.3 Sputum processing

In accordance with the previously described method, sputum sample was hydrolysed using Sputolysin and then serially diluted to 1 in 10,000 times with sterile saline. 100 µL of diluted sputum was plated onto BA plate, CBA plate and PIA plate. Plates were cultured in 37 ˚C for 48 hours, chocolate blood agar was incubated in an aerobic atmosphere enriched with 5-10 % carbon dioxide (Figure 7).

After 48 hours of culture, colony numbers on plates were counted. Three colonies were picked and identified.

2.2.2.4 Throat swabs processing

Swabs were plated on CBA plate, BA plate and PIA plate and then streak using a loop to zone 1, 2, 3 and 4, the loop was sterilised between each zone. Then incubated for 48 hours. After 48 hours, growth was measured as follows: zone 1- light growth; zone 1 and zone 2 - medium growth; zone1, zone 2, zone 3 or even to zone 4 - heavy growth (Figure 14).

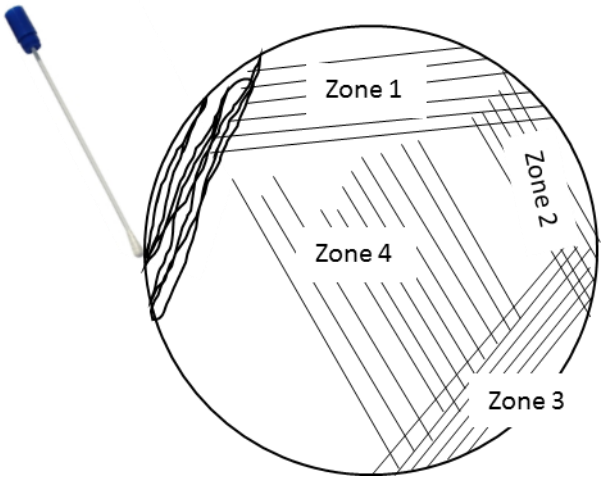


Figure 15. The growth zones for throat swab on CBA plates. Growth was measured as: zone 1- light growth; zone 1 and zone 2 - medium growth; zone1, zone 2, zone 3 or even to zone 4 - heavy growth.

2.2.2.5 Bacteria identification

H. parainfluenzae and NTHi were differentiated by XV test.

2.2.2.6 Bacteria collection

NTHi and *H. parainfluenzae* from both sputum and throat swabs were collected and stored in 10% skimmed milk in -80 °C.

2.2.2.7 *H. parainfluenzae* genomic profile

Genomic profile of *H. parainfluenzae* from same patient's sputum and throat swab were compared using Pulsed-field gel electrophoresis (PFGE).

Materials and equipment

- Low melt agarose (Bio-Rad Laboratories)
- SE Buffer (75 mM NaCl, 25 mM EDTA, pH 7.5)
- Lysis Buffer (1% N-lauroylsarosine, 0.5mM EDTA, pH 9.5)
- Triton X-100 (Sigma®)
- TE Buffer (10mM Tris, 10 mM EDTA, pH 7.5)
- Distilled water
- Reaction buffer (10× React buffer 2, Invitrogen™)
- Bovine serum albumin (BSA 1 mg/mL, New England BioLabs® Inc.)
- Restriction enzyme (XbaI, Invitrogen™)
- Dithiothreitol (DTT 1.5 mg/100 µL in distilled water)
- TBE Buffer (Severn Biotech Ltd.)
- Pulsed-field agarose (Bio-Rad Laboratories)
- Gel red (7.5 µL in 250 mL of 0.1M NaCl)
- CHEF-DRII PFGE system (Bio-Rad Laboratories)

Methods

- Plug preparation

Fresh *H. parainfluenzae* bacterial cells grown for 24 hours at 37 °C on CBA plates were suspended in 1 mL SE buffer, in Eppendorf tubes and centrifuged for 2 minutes

at 13,000 rpm. The pellets were re-suspended in 500 µL of SE buffer but the volume was adjusted depending on the size of the pellet to give a uniform cell density for each isolate. The bacterial cells were encased in an agarose matrix made up of 1.5% low melt agarose in SE buffer (0.15 g of low melt agarose in 10 mL of SE buffer), previously microwaved for 15 seconds and then 4 times 5 seconds, gently mixing between every heating. An equal volume of 1.5% low melt agarose solution was added to the bacterial suspension in SE buffer and subsequently pipetted into the plug mould. Plugs were left at 4 °C for 15 minutes to solidify. Plugs were then transferred into small bijoux containing 2 mL of lysis buffer and 10 µL Triton X-100 and incubated overnight in a water bath at 55 °C. Plugs were then washed by removing the lysis buffer and replacing it with 2 mL of TE buffer for 30 minutes at 4 °C and repeating this step carefully another two times to ensure removal of any potential cellular debris that would prevent proper digestion in later steps.

- Restriction enzyme digestion

A small portion of each plug of approximately 2 mm was cut and placed in an Eppendorf tube filled with 90 µL of distilled water and 10 µL of reaction buffer and placed in a fridge at 4 °C for 30 minutes. The buffer was carefully removed using a fine pasteur pipette and replaced by a solution containing 90 µL of distilled water, 10 µL of reaction buffer, 2 µL of BSA, 2 µL of dithiothreitol and 2 µL of restriction enzyme in which the plugs were incubated overnight at 37 °C in a water bath.

- Loading of plug slices and running of the gel

2 L of 0.5% TBE buffer was made and 150 mL of it was used for making the gel, to which 1.5 g of pulsed-field agarose was added and the mix was microwaved for 1 minute, then mixed and again microwave for 1-minute and10 seconds. Once cooled, the agarose was poured into the gel mould previously cleaned with ethanol, and allowed to solidify for 30 minutes. Finally, the plugs were placed in the wells and the gel was placed in the CHEF-DRII gel tank and the remainder of the 2 L of TBE buffer was added. The chiller was set at 14 °C and parameters were set as follows: initial time 0.5 seconds, final time 20 seconds, voltage 6 volts/cm, run time 16 hours.

- Image analysis

The gel was stained with gel red for visualization under UV trans-illumination using the Molecular Imager® GelDoc™ XR+ system with Image Lab™ software (Bio-Rad Laboratories). The exposure time and saturation were adjusted to obtain optimal pictures with distinct bands.

2.2.2.8 16HBE cells co-culture with *H. parainfluenzae* from upper airways and lower airways

Inflammatory effect of *H. parainfluenzae* from same patient's sputum and throat swab sample on 16HBE cell line were tested. 10^7 cfu/mL *H. parainfluenzae* from patient sputum and 10^7 cfu/mL *H. parainfluenzae* from same patient throat swab were co-cultured with 16HBE cells for 8 hours. Control group was the same volume of phosphate buffered saline (PBS) co-cultured with 16HBE cells for 8 hours. After 8 hours, spin plates at 300×g for 5 minutes, then abstracted supernatant. Supernatant were stored in -20 °C.

2.2.2.9 Cell pro-inflammatory response measurement

Supernatant CXCL8 and CXCL1 were measured using ELISA in accordance with the manufacture instruction.

CXCL8 ELISA assay

Materials and equipment

- PBS (Gibco®)
- Human CXCL8 Duoset ELISA kit® contains standards, detection antibody and streptavidin-HRP. (R&D System®)
- Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4
- Block Buffer: 1% BSA (Sigma®) in PBS
- Reagent Diluent: 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline
- Substrate Solution: 1:1 mixture of 0.75mM H_2O_2 (Sigma®)
- Tetramethylbenzidine (TMB) (Sigma®)
- Stop solution: 2 mM H_2SO_4
- 96 well microplates

- Plate reader

Methods

CXCL8 ELISA was performed according to manufactures instructions, the main steps were: 96-well microplate was coated with capture antibody and left overnight at room temperature. The next day, each well was aspirated and washed with Wash Buffer three times. After the final wash, all the wash buffer was removed and the plate dried, 300 μL of Block Buffer was added to each well for more than 1 hour. At the same time, serum samples were defrosted at room temperature, then vortexed and diluted 20 times with reagent diluent. Standards were serially diluted with reagent diluent. The plate was washed three times after 1 hour and then it was ready for sample culturing. For sample culturing, 100 μL of diluted sample or standards were added to plate in duplicate, appropriate diluent was added, plate covered and cultured at room temperature for 2 hours. After 2 hours, plate was washed, then 100 μL of the working dilution of Detection Antibody was added to each well, plate covered and incubated at room temperature for 2 hours. The plate was washed and 100 μL of the working dilution of Streptavidin-HRP was added and incubated in the dark for 20 minutes. Repeated washing, 100 μL of Substrate Solution was added to each well and incubated in the dark for 20 minutes, then 50 μL of stop solution was added to each well, and the plate was read at 450 nm and 570 nm wavelengths using plate reader.

2.2.2.10 Validation of ELISA

All ELISA assays were validated. Three parameters were assessed in validating assays: Reliability, recovery of spiked mediators and the effect of sample dilution.

Reliability: The reliability of each ELISA assay was determined by assessing their intra-assay co-efficient and inter-assay coefficient of variation. Individual samples were assayed 5 times on a single plate to obtain the intra-assay coefficient of variation. Each sample was also assayed 5 times on different plates to obtain the inter-assay coefficient of variation.

Spike and recovery: a known quantity of each mediator was spiked into the 3 samples. These “spiked” samples were then assayed and compared to the values obtained for

the original sample. The obtained value was divided by the predicted value to calculate the recovery percentage.

Linearity-of dilution effect: The three samples were assayed at dilutions ranging from 1 in 1, to 1 in 5. Observed values were assessed relative to the assay standard curve.

A linearity-of-dilution experiment provides information about the precision of results for samples tested at different levels of dilution in a chosen sample diluent. If the linearity is good over a wide range of dilutions, then the assay method provides flexibility to assay samples with different levels of dilutions.

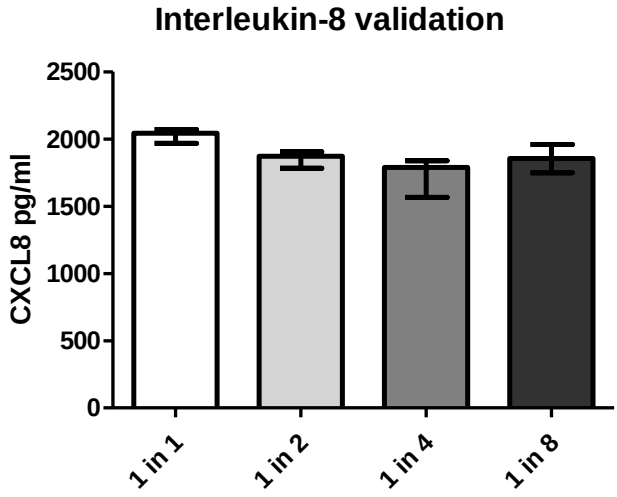
An assay was deemed to be valid if it had a reliable standard curve, an intra-assay and inter-assay coefficient of variation <10%, recovery of spiked samples in the range 80-120% of predicted and a linear dilution effect (Stockley and Bayley 2000). All ELISA assay passed each validation step and was used in the study. Each assay gave a linear, reliable standard curve and representative standard curves are shown below.

Table 8 shows the validation data for all ELISA assays tested. The figure below shows the validation data for CXCL-8. Figure 15 shows the linearity of the dilution effect. Figure 16 shows the representative standard curve for measurement of interleukin 8.

Table 8 Validation of ELISA assays used in the study

	CXCL8	CXCL1	ICAM1	LCN2	MPO	IL1β
Spike-recovery (median%-IQR)	92%(89- 94)	96%(94- 96)	98%(96- 98)	101%(94- 110)	100%(90- 104)	99%(95- 103)
intra assay co-efficient	5.60%	2.30%	5.80%	4.10%	4.20%	5.50%
inter assay co-efficient	7.20%	7.15%	5.40%	6.70%	3.90%	7.80%

a)



b)

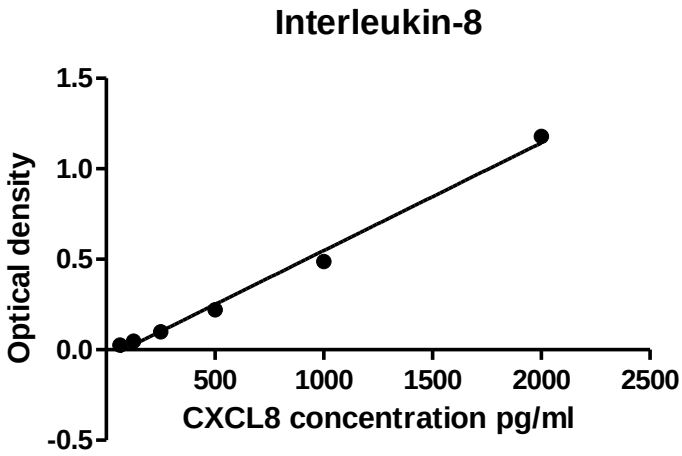


Figure 16. Validation of ELISA assays for CXCL8 measurements: a) linearity of dilution effects interleukin-8. Samples were diluted to 1 in 1, 1 in 2, 1 in 4 and 1in 8. Then CXCL8 level was measured and calculated according to the dilutions. b) representative standard curve for sputum measurement of interleukin-8. R^2 =0.9924

2.2.3 Compare clinical features of *H. parainfluenzae* and NTHi in bronchiectasis patients

2.2.3.1 Sputum sol preparation

Sputum was centrifuged at 50,000×g for 90 minutes to get sputum sol phase for the measuring of airways inflammation. It was stored at -80 ˚C.

2.2.3.2 Sputum inflammation

For sputum inflammation, sputum sol MPO was measured.

MPO test

Materials and equipment

- MPO (Sigma®)
- PBS (Gibco®)
- 0.75 mM H_2O_2
- TMB (Sigma®)
- 2 mM H_2SO_4
- 96 well plates
- Plate reader with a wavelength of 450 nm

Methods

Sputum sol samples were diluted 50 times in PBS, 10 μL diluted sample and standard were reacted with 80 μL 0.75 mM H_2O_2 and 110 μL TMB in 96 well plates for 5 minutes at 37 ˚C. The reaction was stopped by addition of 50 μL 2 M H_2SO_4. The plate was read in a plate reader at a wavelength of 450 nm. Serial dilutions of MPO (33 μg/mL) were used as standards. Samples and standards were all tested in duplicate.

2.2.3.3 Blood processing

Blood samples were taken from patients in the hospital and then centrifuge for 15 minutes at 1000×g. Serum was pipetted off and stored at -80 ℃ before further tests.

Blood samples were sent to the NHS microbiology laboratory for WCC, ESR and CRP tests.

2.2.3.4 Serum inflammation

Serum WCC, ESR, CRP results were from NHS trust laboratory, serum ICAM-1 was measured by ELISA.

Serum ICAM-1 test

Materials and equipment

- PBS (Gibco)
- Human ICAM-1 Duoset ELISA kit® contains standards, detection antibody and streptavidin-HRP. (R&D System®)
- Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4
- Reagent Diluent: 1% BSA (Sigma®) in PBS
- Substrate Solution: 1:1 mixture of 0.75 mM H_2O_2 (Sigma®)
- TMB (Sigma®)
- Stop solution: 2 mM H_2SO_4
- 96 well microplates
- Plate reader

Methods

ICAM-1 ELISA was performed according to manufactures instructions, the main steps were: 96 well microplate was coated with capture antibody and left overnight at room temperature. The next day, each well was aspirated and washed with Wash Buffer three times. After the final wash, all the wash buffer was removed and the plate dried, 300 μL of Reagent Diluent was added to each well for more than 1 hour. At the same time, serum samples were defrosted at room temperature, then vortexed and diluted 20 times with reagent diluent. Standards were serially diluted with reagent diluent. The

plate was washed three times after 1 hour and then it was ready for sample culturing. For sample culturing, 100 µL of diluted sample or standards were added to plate in duplicate, appropriate diluent was added, plate covered and cultured at room temperature for 2 hours. After 2 hours, plate was washed, then 100 µL of the working dilution of Detection Antibody was added to each well, plate covered and incubated at room temperature for 2 hours. The plate was washed and 100 µL of the working dilution of Streptavidin-HRP was added and incubated in the dark for 20 minutes. Repeated washing, 100 µL of Substrate Solution was added to each well and incubated in the dark for 20 minutes, then 50 µL of Stop Solution was added to each well, and the plate was read at 450 nm and 570 nm wavelengths using plate reader.

2.2.4 Assess impact of *H. parainfluenzae* on epithelial cells

Inflammatory effect of *H. parainfluenzae*, NTHi and *P. aeruginosa* on 16HBE cell lines, human primary bronchial epithelial cells and bronchiectasis patient primary nasal epithelial cells were tested.

2.2.4.1 16HBE cell culture

Materials and equipment

- Dulbecco's Modified Eagle Medium (DMEM) (Gibco®)
- 10% fetal bovine serum (Gibco®)
- 1% penicillin and streptomycin (Gibco®)
- 1% L-glutamine (Gibco®)
- 37 ˚C, 5% CO_2 incubator

Methods

16HBE cells are human bronchial epithelial cell line, 16HBE cells were cultured in DMEM, containing 10% fetal bovine serum, 1% penicillin and streptomycin, 1% L-glutamine. Cells were kept in 37 ˚C, 5% CO_2 incubator. For cell infection experiment, cells were seeded into 12 wells plate with each well containing 5×10^6 cells. Plates were incubated at 37 ˚C for 48 hours, then medium extracted, and wells washed with PBS twice. Penicillin and streptomycin free medium were added and cells were ready for infection experiments.

2.2.4.2 Human Bronchial Epithelial Cells (NHBE) culture

Material and equipment

- NHBE (Lonza™)
- BEBM™ Bronchial Epithelial Cell Growth Basal Medium (Lonza™)
- BEGM™ SingleQuots™ Supplement Pack (Lonza™)
- Trypsin/EDTA (Lonza™)
- Trypsin neutralising solution (Lonza™)
- Hepes solution (Lonza™)
- 12 wells plates
- 37 ˚C, 5% CO_2 incubator

Methods

NHBE from Lonza™ was isolated from the epithelial lining of the airways above bifurcation of the lungs of normal donors, cells cultured in BEBM™ Bronchial Epithelial Cell Growth Basal Medium with BEGM™ SingleQuots™ Supplement Pack including: 0.4% Orange Cap Vial with BPE, 0.1% Lilac Cap Vial with Insulin, 0.1% Natural Cap Vial with Hydrocortisone, 0.1% Red Cap Vial with GA-1000, 0.1% Amber Vial with Retinoic Acid, 0.1% Natural Cap Vial with Transferrin, 0.1% Amber Vial with Triiodothyronine, 0.1% Amber Vial with Epinephrine and 0.1% Green Cap Vial with hEGF. Cells were kept in 37 ˚C, 5% CO_2 incubator. Media was changed every two days. For cell infection experiment, cells were seeded into 12 wells plate, with each well containing 5×10^5 cells. Cells remained at 37˚C until fully confluent. Then cells were ready for infection.

2.2.4.3 Human primary nasal epithelial cells culture

Nasal cells were isolated from bronchiectasis patients.

A. Inclusion criteria of bronchiectasis patients

Patients were selected from the bronchiectasis clinic in the Royal Infirmary of Edinburgh. Inclusion criteria for the stable patients included: patients with clinically significant bronchiectasis and don not have severe asthma; 18 years old and older; have not had an exacerbation for at least 4 weeks; were not on long term oral or inhaled antibiotics; were not rhinitis on nasal steroids; were not on anticoagulants; not current smokers or ex-smoker less than 1 year; not currently enrolled in a clinical trial of an investigation medical product within the or 28 days prior.

B. Preparation before sample collecting (Collagen coating of 6 well plates)

Materials and equipment

- PureCol® Collagen (5409, Nutacon, Netherlands)
- Tissue culture grade water
- PBS (Gibco®)
- 6 wells plates
- 4 °C fridge

Methods

Added 670 µL of PureCol Collagen to 50 mL tissue culture grade water and mixed well. Used this collagen within 24 hours and stored at 4 °C. For coating the plates, 3750 µL/well of prepared collagen solution was added and plates were left in the hood with the plate lids off to evaporate. Removed remaining collagen and washed wells once in PBS before cell culture.

C. Cell collection and culture

Materials and equipment

- Sterile endocervical brushes
- BEBM™ Bronchial Epithelial Cell Growth Basal Medium (Lonza™)

- BEGM™ SingleQuots™ Supplement Pack (Lonza™)
- Trypsin/EDTA (Lonza™)
- Trypsin neutralising solution (Lonza™)
- Hepes solution (Lonza™)
- Collagen coated plates
- 12 well plates
- 37 ˚C, 5% CO_2 incubator

Methods

Cells were collected from brushing patient nasal cavity with a sterile brush. Then twirled brush in 1mL warm BEGM media. The media need to be kept warm. As soon as cells were collected, they were divided into 2 wells in collagen-coated plates. This process needed to be done as quickly as possible, ideally in 5 minutes. Cells were cultured in 37 ˚C, 5% CO_2 incubator.

After two days, the cells were checked to ensure they had stuck to the bottom of the well before feeding fresh media. If not, only half the media was removed and replaced with fresh media. The removed media was seeded into a second collagen-coated plate to encourage further attachment and growth.

After another 48 hours, if there was evidence of cell attachment, the supernatant and unattached cells were removed and replaced with fresh BEGM, cells were fed every 2-3 days. When cells were confluent they were split into a new uncoated plate for experiments. For cell infection experiment, cells were seeded into 12 wells plate, with each well containing 5×10^5 cells. Cells were kept at 37 ˚C until fully confluent.

2.2.4.4 Bacterial preparation

Six different strains of unselected *H. parainfluenzae*, NTHi and *P. aeruginosa* from previously-stored bronchiectasis patient colonies collection.

Materials and equipment

- CBA plates
- Nutrient agar plates

- 5% CO_2 incubator
- 37 ˚C incubator

Methods

NTHi and *H. parainfluenzae* were grown on CBA plates, incubated at 37 ˚C in 5% CO_2 overnight. *P. aeruginosa* were grown on nutrient agar, at 37 ˚C overnight.

2.2.4.5 Equation of bacterial optical density and bacterial load (cfu/mL)

The experiments below are key so that an accurate bacterial load can be derived for the different pathogens. Correlation of *H. influenzae* and *H. parainfluenzae* OD value and colony forming unite have been tested to get an accurate bacterial load (Figure 16).

Materials and equipment

- CBA plates
- Nutrient agar
- PBS
- Spectrophotometer
- 5% CO_2 incubator
- 37 ˚C incubator

Methods

Bacteria (*H. parainfluenzae*, NTHi and *P. aeruginosa*) were cultured on agar plates overnight. Next day, colonies were picked from the plate and then suspended in PBS. The suspensions were diluted with PBS: 1 in 1, 1 in 2, 1 in 3, 1 in 4, 1 in 10, 1 in 20 and 1 in 100. OD value was measured for each dilution, and each dilution was also plated on an agar plate and cultured for colony number counting next day. The equations were used to calculate bacterial load when cells were co-cultured with bacteria. The correlations have been proved significant before each test. Figure 17 show the equation of *H. parainfluenzae*, NTHi and *P. aeruginosa* OD value and bacterial load (cfu/mL).

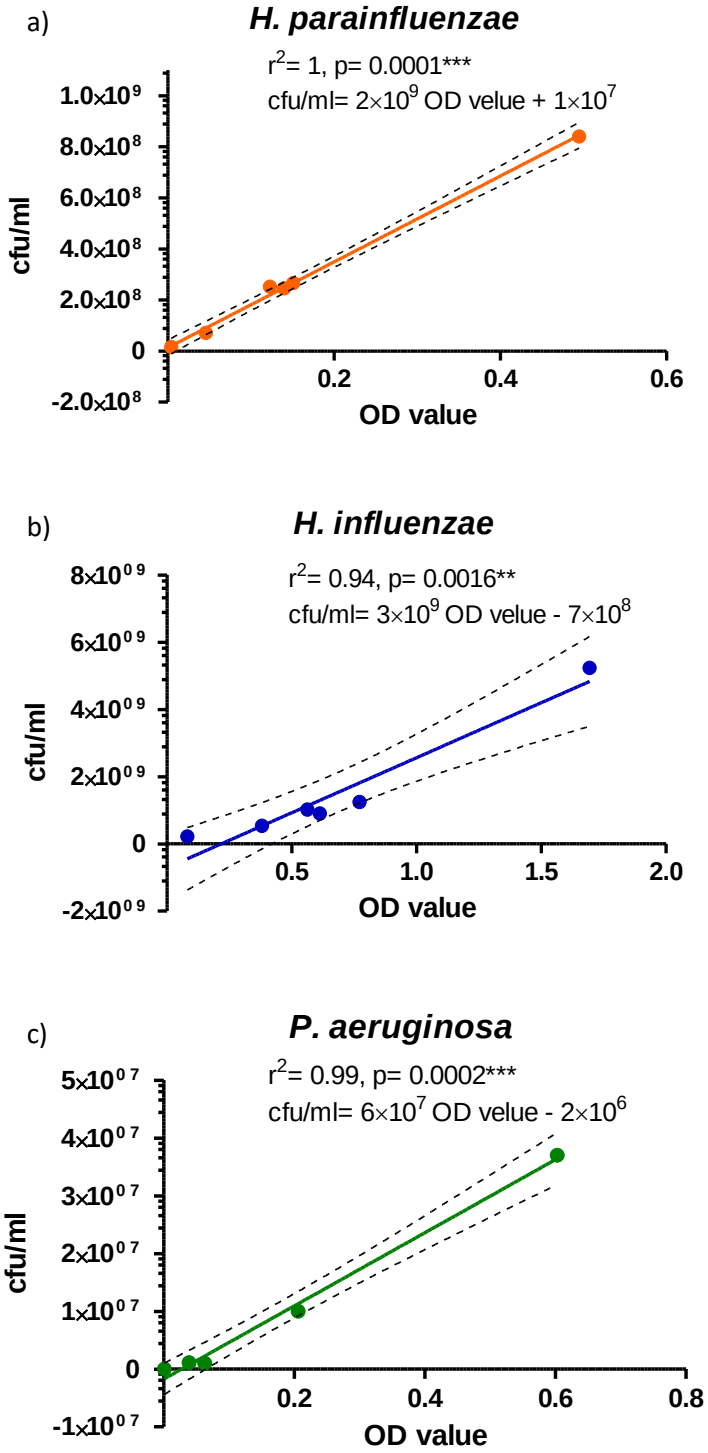


Figure 17. Equation of bacterial optical density and bacterial load (cfu/mL). a) is the correlation between the *H. parainfluenzae* cfu and OD value. The equation between *H. parainfluenzae* OD value and cfu is cfu $=2\times10^{9}\times$OD value $+1\times10^{9}$. b) is the correlation between the NTHi cfu and OD value. The equation between NTHi OD value and cfu is cfu $=3\times10^{7}\times$OD value -7×10^{8}. c) is the correlation between the *P. aeruginosa* cfu and OD value. The equation between *P. aeruginosa* OD value and cfu is cfu$=6\times10^{7}\times$OD value -2×10^{6}.

3.2.4.6 Heat killed bacterial preparation

Materials and equipment

- 80 ˚C water bath
- CBA plates
- Nutrient agar plates

Methods

Bacterial suspensions, in sterile PBS, of *H. parainfluenzae* (n=6), NTHi (n=6) and *P. aeruginosa* (n=6) were prepared, using colonies from agar plates. Then OD value of the bacterial suspension was measured at a wavelength of 600 nm. Then the bacterial load was calculated by using the previously calculated equation. Bacteria, at the required bacterial load, were heat-killed by placing them into an 80 ˚C water bath for an hour before co-cultured with cells. 100 µL of heat-treated bacteria were plated on an agar plate and cultured overnight to check the viability. All heat-treated bacteria showed no bacterial growth after the overnight incubation.

2.2.4.7 Epithelial cells co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa*

Materials and equipment

- Sterile PBS
- Spectrophotometer
- Centrifuge

Methods

Bacterial suspensions, in sterile PBS, of *H. parainfluenzae*, NTHi and *P. aeruginosa* were prepared, using colonies from agar plates. OD_{600} value was then measured and the bacterial load was calculated based on the previously mentioned equation (Figure 10). Bacteria were then diluted to the required bacterial load. 100 µL bacteria were co-cultured with cells in duplicate for various experiments. The negative control contained 100 µL PBS co-cultured with cells. For time-course experiments, 10^7 cfu/mL bacteria were co-cultured with cells for 1hour, 2 hours, 4 hours, 6 hours and 8 hours. For bacterial dose-response experiments, 10^3-10^8 cfu/mL bacteria were co-cultured with cells for 8 hours.

After co-culture, cell culture media was pipetted off, centrifuged at 300×g for 5 minutes, then the supernatant was removed and stored at -20˚C.

2.2.4.8 Cell lactate dehydrogenase release after co-culture

Cell lactate dehydrogenase (LDH) release was measured in previously collected cell culture supernatant. LDH commonly exist in living cells. When cell membranes are damaged, LDH is released to the surrounding environment. Cytotoxicity Detection Kit was used.

Materials and equipment

- Cytotoxicity Detection Kit contains catalyst and dye solution (Sigma-Aldrich)®
- Plate reader with wavelength of 490 nm and 600 nm

Methods

LDH assay was performed according to the manufacturer's instructions. To determine the LDH activity in media samples, samples were defrosted to room temperature. Fill the well with 100 µL sample, all samples were tested in duplicate. 100 µL freshly prepared Reaction mixture (for 100 tests, mix 250 µL Catalyst with 11.25 mL dye solution) added to each well and incubated for up to 30 minutes at 15 °C to 25 °C. During this incubation period, the plate should be protected from light. The absorbance of the samples was measured at 490 nm and 600 nm.

2.2.4.9 Epithelial cells pro-inflammatory response measurement

Cytokines produced by cells after co-cultured with bacteria were tested. Cytokine profile (105 cytokines show in Table 7) was measured using Proteome Profiler Human XL Cytokine Array kit®. For quantitative CXCL8, IL1β, LCN2, CXCL1 and ICAM-1 measurement, Duoset ELISA kit® was used.

A. Epithelial cells cytokine profile measurement

Materials and equipment

- Distilled water
- Proteome Profiler Human XL Cytokine Array Kit (R&D system) contains:
  - Human XL Cytokine Array
  - Array Buffer 4
  - Array Buffer 6
  - Wash Buffer Concentrate
  - Detection Antibody Cocktail, Human XL Cytokine Array
  - Streptavidin-HRP
  - Chemi Reagent 1
  - Chemi Reagent 2
- 4-Well Multi-dish
- Transparency Overlay Template
- Rocking platform shaker
- Autoradiography cassette
- Film developer
- X-ray film (Kodak® BioMax™ Light-1)

Methods

- Sample preparation

Cells were co-cultured with four groups of bacteria:

- 10^4 cfu/mL *H. parainfluenzae* (6 different clinical strains)
- 10^7 cfu/mL *H. parainfluenzae* (same strains as the first group)

- 10^7 cfu/mL NTHi (6 different clinical strains)
- 10^7 cfu/mL *P. aeruginosa* (6 different clinical strains)

After 8 hours, cell culture media was collected and stored as previously described. Before the array test, all samples were defrosted to room temperature. 100 µL were abstracted from each sample then pooled with the other five samples from the same group. These four pooled samples were used for further tests.

- Proteome Profiler Human XL Cytokine Array kit

Proteome Profiler Human XL Cytokine Array is a membrane-based antibody array for the parallel determination of the relative levels of selected human cytokines and chemokines. There are four membranes spotted with 105 different antibodies to human cytokines (Table 7).

The method strictly followed the instruction from the Proteome Profiler Human XL Cytokine Array Kit. The main steps are:

Placed each membrane in a separate well and block with 2 mL of Array Buffer 6. Incubated for one hour on a rocking platform shaker. While the arrays were blocking, prepared the pooled sample to a final volume of 1.5 mL with Array Buffer 6. Array Buffer 6 was aspirated from the wells of the 4Well Multi-dish and the prepared samples added. Incubated overnight at 2-8 °C on a rocking platform shaker.

The next day, washed each membrane with 1×Wash Buffer for 10 minutes on a rocking platform shaker three times. Returned the array to the 4 Well Multi-dish containing the diluted Detection Antibody Cocktail (30 µL of Detection Antibody Cocktail to 1.5 mL of 1× Array Buffer 4/6), and covered with the lid. Incubated for 1 hour on a rocking platform shaker. Repeated the washing step, then returned the membrane to the 4-Well Multi-dish containing 1.5mL 1× Streptavidin-HRP. Covered the wells with the lid. Incubated for 30 minutes at room temperature on a rocking platform shaker. Washed each array again, then pipetted 1.0 mL of the prepared Chemi Reagent Mix evenly onto each membrane. Carefully covered with the top sheet of the plastic sheet protector. Gently smoothed out any air bubbles and ensured Chemi Reagent Mix was spread evenly to all corners of each membrane. Incubated for 1 minute. Positioned paper

towels on the top and sides of the plastic sheet protector containing the membranes and carefully squeezed out excess Chemi Reagent Mix. Then covered the membranes with plastic wrap. Placed the membranes with the identification numbers facing up in an autoradiography film cassette. Exposed membranes to X-ray film for 30 seconds, 1 minute, 3 minutes and 5 minutes.

- DATA analysis

X-ray films were scanned and the density of individual spot was calculated using software Image J®. A template was provided with the kit.

Table 9. 105 cytokines measured in micro array

Growth factors	Tumor necrosis factor family	Immunoglobulin superfamily	Chemokines	Hematopoietin family	Interferon family	Interleukin 1 family	Others	Others
Angiogenin	TNF-alpha	EMMPRIN	CXCL1/GRO alpha	IL-6	IL-24	IL-17A	Lipocalin-2	CD14
HGF	CD40 Ligand	ICAM-1/CD54	IL-8	GM-CSF	IL-22	IL-1 alpha/IL-1F1	uPAR	Adiponectin/Acrp30
FGF basic	Fas Ligand	RAGE	CCL20/MIP-3 alpha	IL-5	IFN-gamma	IL-1 beta/IL-1F2	Adiponectin	Cripto-1
LIF	BAFF	CD31	CCL2/MCP-1	IL-4	IL-10	IL-1ra/IL-1F3	Endoglin	Cystatin C
Angiopoietin-1	CD30		CXCL10/IP-10	IL-23	IL-19	ST2/IL1 R4	MMP-9	Dkk-1
BDNF			CXCL11/I-TAC	M-CSF		IL-2	CHI3l1	DPPIV/CD26
Angiopoietin-2			CCL7/MCP-3	IL-27		IL-18 BPa	CRP	EGF
KGF/FGF-7			CXCL9/MIG	IL-11		IL-33	FGF-19	Flt-3 Ligand
GDF-15			CCL3/CCL4	IL-12 p70			apolipoprotein A-1	IGFBP-2
PDGF-AA			CCL19/MIP-3 beta	IL-13			TfR	IGFBP-3
PDGF-AB/BB			CXCL4/PF4	IL-15			Vitamin D BP	IL-16
TGF-alpha			CCL5/RANTES	IL-31			Kallikrein 3/PSA	IL-32 α/β/γ
VEGF			CXCL12/SDF-1 alpha	Growth Hormone			PDGF-AA	IL-34
			CCL17/TARC	IL-3			MIF	Leptin
			CXCL5/ENA-78	G-CSF			Pentraxin 3/TSF-14	Myeloperoxidase
							SHBG	Osteopontin
							ST2/IL1 R4	RBP4
							TFF3	Relaxin-2
							TIM-3	Resistin
							VCAM-1	Serpin E1/PAI-1

B. Epithelial cells quantitative CXCL8, IL1β, Lipocalin2, CXCL1 and ICAM-1 measurement

Duoset ELISA kit (R&D system) was used according to manufacture as described previously.

2.2.5 Assess the impact of coinfection of *H. parainfluenzae* with *P. aeruginosa* and *H. parainfluenzae* with NTHi

3.2.5.1 *H. parainfluenzae* and *P. aeruginosa* co-cultured with 16HBE cells

A. 16HBE cells were co-cultured with:

- 100 µL 1×10^5 cfu/mL *H. parainfluenzae* and 100 µL PBS
- 100 µL 2×10^5 cfu/mL *H. parainfluenzae* and 100 µL PBS
- 100 µL 1×10^5 cfu/mL *P. aeruginosa* and 100 µL PBS
- 100 µL 2×10^5 cfu/mL *P. aeruginosa* and 100 µL PBS
- 100 µL 1×10^5 cfu/mL *H. parainfluenzae* and 100 µL 1×10^5 cfu/mL *P. aeruginosa*
- 200 µL PBS

After 8 hours, for cytokine measurement, plates were centrifuged at 300×g for 5 minutes, then supernatant abstracted. The supernatant was stored at -20 °C for future CXCL1, CXCL8 and LCN2 measurement.

For outer cellular and intercellular bacterial load measurement, media was abstracted and then serial dilutions were made, 100 µL of diluted media was plated on CBA plates and nutrient plates. Plates were incubated at 37 °C for 24 hours and colonies counted.

B. 16HBE cells were also co-cultured with:

- 100 µL 1×10^6 cfu/mL *H. parainfluenzae* and 100 µL PBS
- 100 µL 2×10^6 cfu/mL *H. parainfluenzae* and 100 µL PBS
- 100 µL 1×10^6 cfu/mL *P. aeruginosa* and 100 µL PBS
- 100 µL 2×10^6 cfu/mL *P. aeruginosa* and 100 µL PBS
- 100 µL 1×10^6 cfu/mL *H. parainfluenzae* and 100 µL 1×10^6 cfu/mL *P. aeruginosa*
- 200 µL PBS

After 8 hours, the supernatant was collected as above for CXCL1, CXCL8 and LCN2 test.

2.2.5.2 16HBE cells were co-cultured with:

- 100 µL 1×10^3 cfu/mL to 1×10^7 cfu/mL NTHi and 100 µL PBS

- 100 µL 1×10^3 cfu/mL to 1×10^7 cfu/mL NTHi and 100 µL 1×10^5 cfu/mL *H. parainfluenzae*
- 200 µL PBS

After 8 hours, the supernatant was collected as above for CXCL1 and CXCL8 test.

2.2.5.3 *H. parainfluenzae* and non-PCN *P. aeruginosa* co-culture with 16HBE cell line in combination

16HBE cells were co-cultured with:

- 100 µL 1×10^5cfu/mL *H. parainfluenzae* and 100 µL PBS
- 100 µL 1×10^5cfu/mL non-PCN *P. aeruginosa* and 100 µL PBS
- 100 µL 1×10^5cfu/mL *H. parainfluenzae* and 100 µL 1×10^5cfu/mL non-PCN *P. aeruginosa*
- 200 µL PBS

After 8 hours, the supernatant was collected as above for CXCL1 and CXCL8 test.

2.2.5.4 Heat-treated *H. parainfluenzae* and *P. aeruginosa* co-culture with 16HBE cell line in combination

16HBE cells were co-cultured with:

- 100 µL 1×10^5 cfu/mL heat-treated *H. parainfluenzae* and 100 µL PBS
- 100 µL 1×10^5 cfu/mL heat-treated *P. aeruginosa* and 100 µL PBS
- 100 µL 1×10^5 cfu/mL heat-treated *H. parainfluenzae* and 100 µL 1×10^5 cfu/mL heat-treated *P. aeruginosa*
- 200 µL PBS

2.2.5.5 *H. parainfluenzae* co-culture with 16HBE cell line in the media that has cultured *P. aeruginosa*

16HBE cells were co-cultured with 1×10^5 cfu/mL *P. aeruginosa* for 8 hours, the supernatants were collected and filtered through 0.22 μm^2 filters to remove *P. aeruginosa*, then stored at -20 °C. Next day, 16HBE cells were co-cultured with 1×10^5 cfu/mL *H. parainfluenzae* for 8 hours in 900 µL supernatant collected the previous day. Cytokine measurement and bacterial load counts were performed as described above.

For the comparison group, 16HBE cells were co-cultured without bacteria for 8 hours, supernatants were collected, filtered and then stored in -20 °C. Next day, 16HBE cells were co-cultured with 10^5 cfu/mL *H. parainfluenzae* for 8 hours in 900 µL supernatant collected the previous day.

A reverse experiment has also been done on 16 HBE cells. 16HBE cells were co-cultured with 10^5 cfu/mL *H. parainfluenzae* for 8 hours, supernatants were collected, filtered and stored in -20 °C. Next day, 16HBE cells were co-cultured with 10^5 cfu/mL *P. aeruginosa* for 8 hours in 900 µL supernatant collected the previous day.

2.2.5.6 *H. parainfluenzae* co-culture with *P. aeruginosa* growth broth

P. aeruginosa was cultured in nutrient broth and *H. parainfluenzae* were cultured in BHI broth (containing Fildes extract, Oxoid®) overnight. Next day, *P. aeruginosa* culture broth was collected and filtered with 0.22 μm^2 filters to remove *P. aeruginosa*. *H. parainfluenzae* culture was diluted by PBS to 50 times, and then 100 µL of diluted *H. parainfluenzae* were cultured with 1 mL of the filtered *P. aeruginosa* broth and 4mL BHI broth (containing Fildes extract). For comparison experiment, 100 µL of diluted *H. parainfluenzae* were cultured with 1 mL nutrient broth and 4mL BHI broth (containing Fildes extract). Incubated in 37°C with 5% CO_2 shaker. OD values were measured after 1hour, 2hours, 4 hours, 6 hours, 8 hours and 24 hours.

In vitro, 16HBE cells were co-cultured with 1×10^5 cfu/mL *H. parainfluenzae*, 200 µL filtered *P. aeruginosa* broth culture and 700 µL DMEM media for 8 hours. For comparison experiment, 16HBE cells were co-cultured with 1×10^5 cfu/mL *H. parainfluenzae*, 200 µL nutrient culture broth and 700 µL DMEM media for 8 hours. After 8 hours, abstract media and serial dilutions were done, then plated on CBA plates. Plates were incubated at 37 °C with 5% CO_2 for 24 hours and colonies counted.

2.2.5.7 *H. parainfluenzae* co-culture with media that has cultured *P. aeruginosa* on CBA plates

16HBE cells were co-cultured with 10^5 cfu/mL *P. aeruginosa* for 8 hours, supernatants were collected, filtered with 0.22 μm^2 filters and stored in -20 °C. The next day, *H. parainfluenzae* colonies were streaked all over the nutrient agar. A heated loop was used to make a 0.5 cm diameter well in the centre of the plate (Figure 18). In the experimental group, 100 µL supernatant collected from the previous day was pipetted into this well, and for the positive control, 100 µL 15 ng/mL polymyxin B was added to the well. Plates were incubated at 37 °C with 5% CO_2 for 24 hours, colonies were counted. Polymyxin B can lead to an inhibition of *H. parainfluenzae* growth.

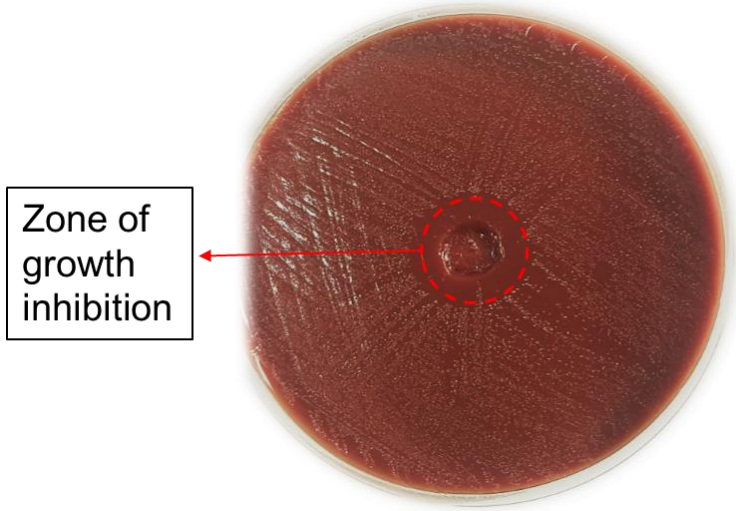


Figure 18. *H. parainfluenzae* co-culture with polymyxin B on CBA plates. A sample of the 0.5 cm diameter well in the centre of the plate and the zone of inhibition caused by 15 ng/mL polymyxin B.

2.2.5.8 Minimum inhibitory concentration (MIC) of Polymyxin B to *H. parainfluenzae*

Five different strains of *H. parainfluenzae* were cultured on CBA plates that contain 0 µg/mL, 3 µg/mL, 5 µg/mL, 8 µg/mL, 10 µg/mL, 15 µg/mL and 30 µg/mL of polymyxin B for overnight. After 24 hours, plates were taken out and the growth of *H. parainfluenzae* was noted down. Table 10 shows the growth of *H. parainfluenzae* on polymyxin B CBA plates. + indicates the growth of *H. parainfluenzae*; - indicates the inhibition of *H. parainfluenzae* growth. The results suggested that at 15 µg/mL, the polymyxin B can lead to inhibition of all *H. parainfluenzae*.

Table 10. Results of *H. parainfluenzae* growth in polymyxin B agar

Polymyxin B	Growth of *H. parainfluenzae*	Growth of control
0 µg/mL	+++++	-
3 µg/mL	+-+++	-
5 µg/mL	--++-	-
8 µg/mL	---+-	-
10 µg/mL	--+--	-
15 µg/mL	-----	-
30 µg/mL	-----	-

2.3 Statistical analysis

Data were present as median and interquartile range (IQR). The difference between two groups was analysed by un-paired Student's t-test or paired t test (GraphPad Prism version 6; GraphPad Software). When three groups were involved, data were analysed by one-way ANOVA with a Bonferroni multiple comparison post-hoc test (GraphPad Prism version 6; GraphPad Software). P-values < 0.05 were considered as statistically significant. The heat map was generated using R®.

Power calculations are carried out using GPower 3.1 Statistical package. For comparing bacterial loads, a 1 log unit change in bacterial load is clinically important. For comparing sputum inflammation, a 33% reduction in sputum myeloperoxidase would represent a significant reduction. Same patients' size was used for comparing serum inflammation.

Bacterial load: With a common standard deviation 0.98 (log cfu/ml), using 5 groups, 90% power and 1.25 % level of significance due to multiple comparisons, 28 per group are needed to detect a 1 log unit change in bacterial load (this represents a major change in bacterial load).

Myeloperoxidase: With a common standard deviation of 0.7 (U/ml), using 5 groups, 90% power and 1.25% level of significance due to multiple comparisons, 24 per group are needed to detect a 33% reduction in sputum myeloperoxidase.

Chapter 3

Results

Chapter 3: Results

3.1 Results for aim 1. Classify *Haemophilus* species in bronchiectasis

This part of the study was done to characterise *H. parainfluenzae*, as well as other common *Haemophilus* species isolated from 140 stable bronchiectasis patients' sputum samples. The most commonly reported *Haemophilus* species in bronchiectasis were tested: NTHi, *H. parainfluenzae* and *H. haemolyticus*. *H. parainfluenzae* subtypes were also investigated.

Sputum samples from another 51 stable bronchiectasis patients were sent to an NHS trusted laboratory to compare outcomes for the isolation of *Haemophilus* species, with special focus on *H. parainfluenzae.*

3.1.1 Bronchiectasis patients recruited

To classify *Haemophilus* species in bronchiectasis patients, 140 patients were recruited. They were chronically colonised but currently stable, with an average age of 69 years old (standard deviation of 11 years). 76 were female, 64 males. 43 were ex-smokers, and 97 non-smokers; there were no current smokers. None of them were on antibiotic treatment since at least four weeks before the study. They all had had *H. influenzae* on their last NHS microbiology laboratory result.

Table 11. Patient's information

Number	140
Sex F/M	76/64
Age	69(21- 91)
Number of ex- smoker (pack year)	43(11)
FEV_1 (L)	1.78(0.6- 3.87)
FEV_1% pred	2.75(1.48- 4.76)
FVC (L)	2.67(1.27- 6.3)
FVC% pred	3.45(1.83- 5.24)
Bronchiectasis lobe number	3(1-6)
Median exacerbation	1(0-12)
Comorbidities (n)	Asthma (60)
	COPD (11)
	ABPA (17)
	IBD (4)
	TB (6)
	Hypertension (24)
	CF (2)
	Gastro-oesphageal reflux disease (9)
	Pulmonary hypertension (7)
Treatment (n)	Inhaled SA B agonists (46)
	Inhaled LA anticholinergics (27)
	Inhaled combined Tx (70)
	Inhaled steroid (23)
	Nebulised bronchodilators (5)

IBD: Inflammatory bowel disease

TB: Tuberculosis

3.1.2 Identification of *H. parainfluenzae,* NTHi and *H. haemolyticus*

Both *H. parainfluenzae* and *H. influenzae* grew on CBA plate. They all formed grey or cream coloured small round colonies. On nutrient agar, *H. parainfluenzae* grew around both V disks, and V+X disks. *H. influenzae* and *H. haemolyticus* grew only around V+X disks. From the *Haemophilus* species collected from those 140 patients, 57 strains were confirmed to be *H. parainfluenzae,* and 66 were identified as either *H. influenzae* or *H. haemolyticus*. These strains were kept at -80 ºC for future experiments.

Forty-five strains of *H. influenzae* (or *H. haemolyticus*) isolated from the last step were recovered from -80 ºC conditions and then tested using qPCR. qPCR results showed 44 strains were *H. influenzae,* and one strain was *H. haemolyticus*.

These 44 *H. influenzae* strains identified in the last step were then confirmed to be NTHi by PCR.

3.1.3 *H. parainfluenzae* was isolated from more than a third of the patients' sputum samples

Among the 140 patients, using traditional microbiology techniques, 57 (40%) patients isolated *H. parainfluenzae* including one patient that had *H. parainfluenzae* and *S. pneumoniae*, and one had *H. parainfluenzae* and *S. aureus*. *H. influenzae* was isolated from 66 (47%) patients including one patient that had *H. influenzae* and *S. pneumoniae* and two had *H. influenzae* and *S. aureus*. Two patients had *H. parainfluenzae* and *H. influenzae*. Furthermore, 1 patient presented *P. aeruginosa*, 2 presented *S. pneumoniae*, and 4 presented *S. aureus*. Out of all patients, 9 (6%) had mixed normal flora (MNF) with no dominant pathogen identified. Using molecular methods, we found one patient presented *H. haemolyticus* and no patients had capsulated *H. influenzae* (Figure 19).

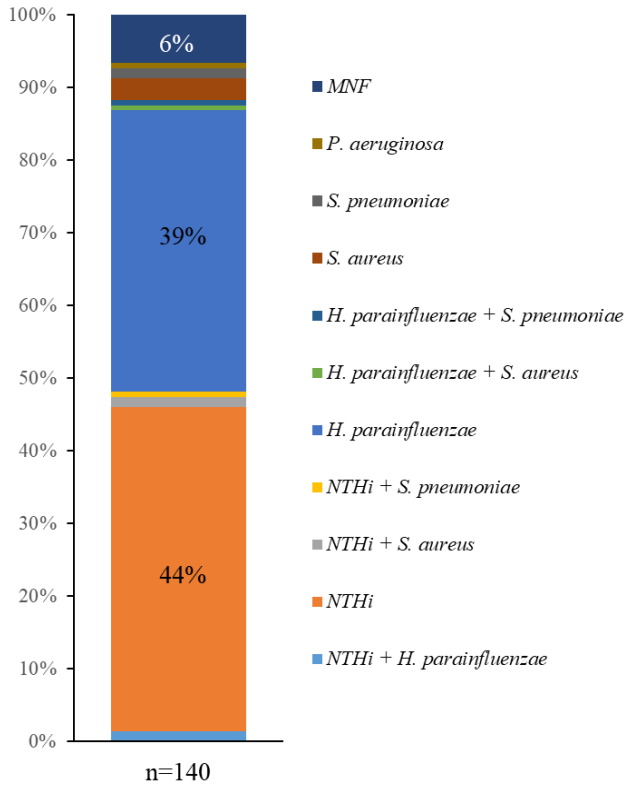


Figure 19. *Haemophilus* species in 140 bronchiectasis patients. 53 (39%) patients had *H. parainfluenzae*. One patient (0.7%) had *H. parainfluenzae* and *S. pneumoniae*, one (0.7%) had *H. parainfluenzae* and *S. aureus*. 61 (44%) patients had NTHi. One patient (0.7%) had *H. influenzae* and *S. pneumoniae*, two (1.4%) had *H. influenzae* and *S. aureus*. Two patients (1.4%) had *H. parainfluenzae* and *H. parainfluenzae*. One patient (0.7%) had *H. haemolyticus*, two (1.4 %) had *S. pneumoniae*, one patient (0.7%) had *P. aeruginosa* and four (2.8%) had *S. a*ureus. Nine (6%) patients had mixed normal flora (MNF) with no dominant pathogen identified.

3.1.4 Subtypes of *H. parainfluenzae* isolated from patients

Subtypes of *H. parainfluenzae* were identified. Forty-one strains of *H. parainfluenzae* were recovered from -80 ºC condition. Most of the *H. parainfluenzae* isolated from patients were subtype V (32%) and subtype I (27%); 15% were subtype II and another 15% subtype III (Figure 20). 7% of the patients had subtype VII, 2% had subtype VIII, and 2% had subtype VIII. There was no patient with subtype VI *H. parainfluenzae*.

Table 12. Differentiation of subtypes of *H. parainfluenzae*

Subtype*	Results of test for production of		
	Indole	Urease	Ornithine decarboxylase
I	-	-	+
II	-	+	+
III	-	+	-
IV	+	+	+
V	-	-	-
VI	+	-	+
VII	+	+	-
VIII	+	-	-

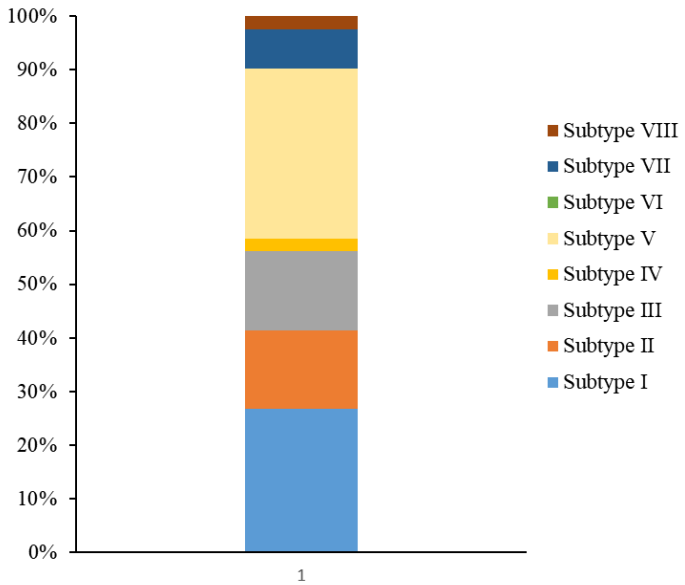


Figure 20. *H. parainfluenzae* subtypes. Forty-one strains of *H. parainfluenzae* were recovered from -80 ºC. Results of indole, urease and ornithine decarboxylase test shows: 11 (27%) strains were subtype I, 6 (15%) strains were subtype II, 6 (15%) strains were subtype III, 1 (2%) strain were subtype IV, 13 (31%) strains were subtype V, there was no subtype VI, 3 (7%) strains were subtype VII, 1 (2%) was subtype VIII.

3.1.5 NHS microbiology laboratory results compared to our laboratory results

Fifty-one unselected patients' sputum microbiology results were compared between the ones obtained in our own laboratory with our proposed method and the ones given by an NHS microbiology laboratory. These patients' samples were collected from the Royal Infirmary bronchiectasis clinic. But they should be ≥ 18 years old, at a stable stage of bronchiectasis, and not on long term oral or inhaled antibiotics. Results from both laboratories showed a distinct difference in the number of pathogens. *H. parainfluenzae* was not reported by the NHS microbiology laboratory. However, our laboratory results showed that 9 patients (18%) had *H. parainfluenzae*. There was higher number of NTHi identified by our laboratory (33%) compared to the NHS microbiology results (20%). We also found less MNF (6%) compared to NHS results (33%) (Figure 21).

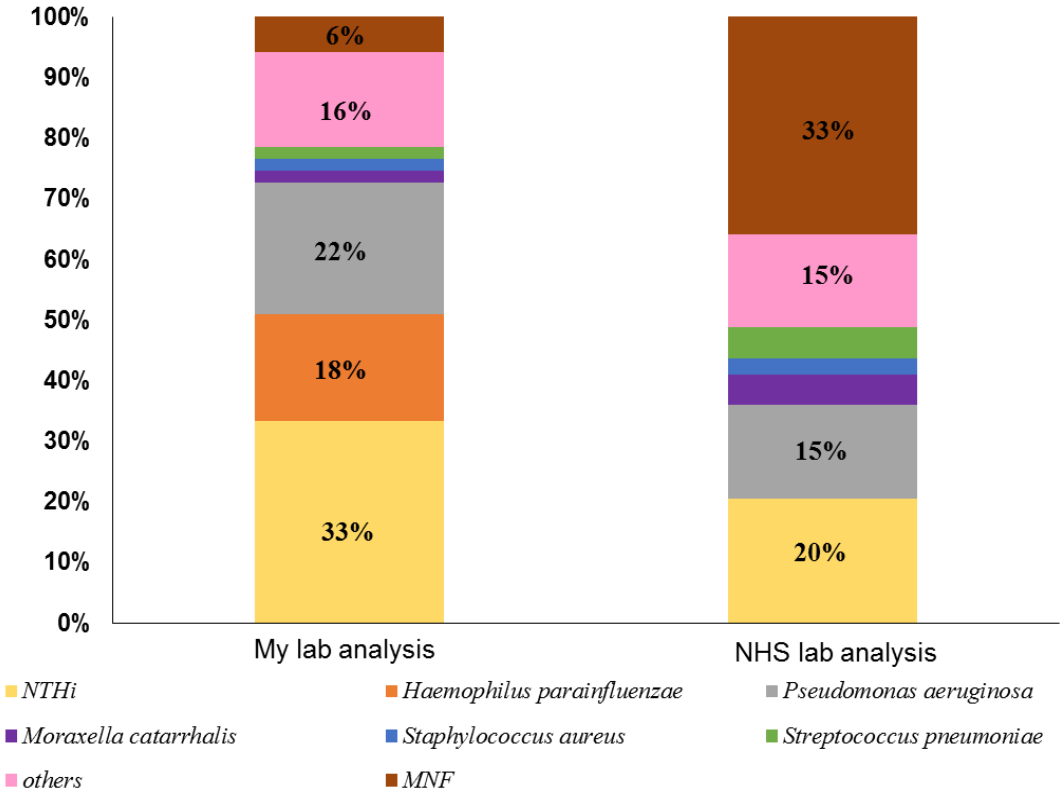


Figure 21. Microbiology results from NHS microbiology laboratory and our laboratory. From my lab results, 17 (33%) patients had NTHi, 9 (18%) patients had *H. parainfluenzae.* 11 (22%) patients had *P. aeruginosa*. One (2%) patient had *Moraxella catarrhalis,* one had *S. aureus* and one had *S. pneumonia.* There 8 (16%) patients had other pathogen such as*:* one *Achromabacter xylosidans,* one *Chryseobacterium indologens,* one *Pasteurella mucocida,* Four *S. maltophilia and* one *E. coli.* Three patients had mixed normal flora. From NHS microbiology results, 10 (23%) patients had NTHi, 0 (0%) patients had *H. parainfluenzae.* 8 (15%) patients had *P. aeruginosa*. Three (5%) patient had *Moraxella catarrhalis,* one (3%) had *Staphylococcus aureus* and three (5%) had *S. pneumoniae.* There were 8 (15%) patients had other pathogens such as: two *Pasteurella multocida, one Klebsiella oxytoca, mycobacteria,* three *S. maltophilia, one Enterococcus faecalis* and one yeast. 17(33%) patients had MNF.

3.2 Results for aim 2. Compare *H. parainfluenzae* isolates from bronchiectasis patients' upper airways and lower airways

Throat swabs have been collected from 30 unselected patients together with their sputum samples. Those patients were from the previously described 140 stable bronchiectasis patients. They were ≧18 years old; have not had any exacerbation for at least 4 weeks before the study; were not on long term oral or inhaled antibiotics; had grown *H. influenzae* in the last sputum culture within the past one year according to NHS microbiology reports.

Eight patients had *H. parainfluenzae* in both throat swab and sputum samples. The *H. parainfluenzae* from same patient's upper airways and low airways were compared for their genomic profile and inflammatory effect on 16HBE cells.

3.2.1 Genomic profile of *H. parainfluenzae* from the upper airways and lower airways

Four pairs of *H. parainfluenzae* from four patients' upper and lower airways were recovered from frozen stock and compared for their genomic profile. The genomic profile of the *H. parainfluenzae* from the same patient's upper and lower airways was different but related (Figure 22). For patient 1, there were four bands different between *H. parainfluenzae* from sputum and throat swab; for patients 2, there were two bands different; for patients 3, there were eight bands different; for patients 4, there were five bands different. Because most pairs had less than 6 bands different, it suggests that *H. parainfluenzae* from upper airways and lower airways are different and possibly related.

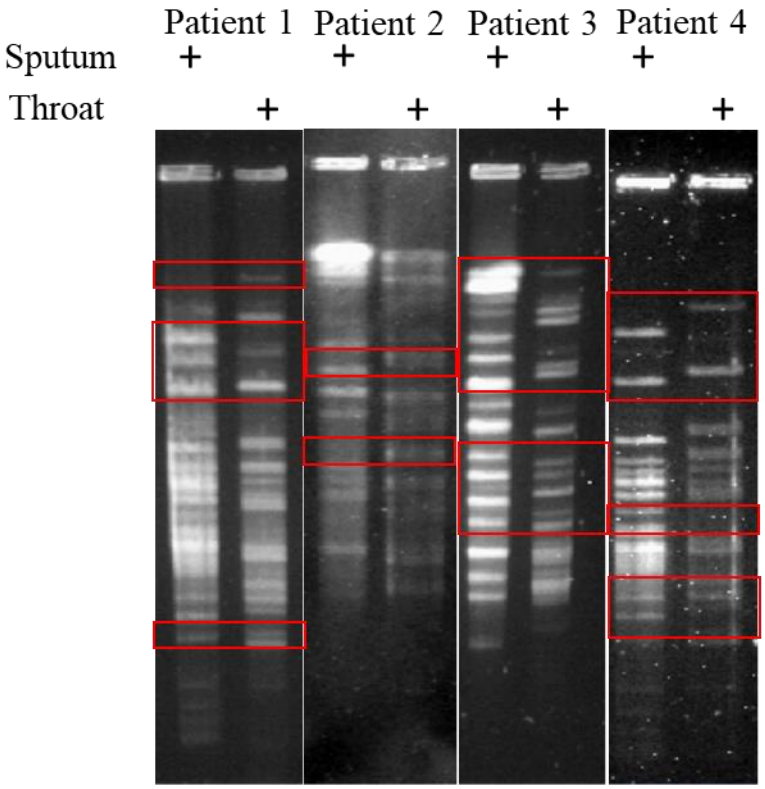


Figure 22. **Genomic profile of *H. parainfluenzae* from patients' upper airways and lower airways.** Four pairs of *H. parainfluenzae* from four individual patients' throat and sputum were compared for their genomic profile using PFGE. The red squares point out the different bands between *H. parainfluenzae* from throat or sputum. For PFGE, when different band number is less than 1, it indicates the genomic profiles are the same; when the number is 1 to 3 (including 3), it indicates that the genomic profiles are closely related; when the number is 4 to 6 (including 6), it indicates the genomic profiles are possible related, when the number more than 6, it indicates the genomic profiles are different.

3.2.2 *H. parainfluenzae* isolated from upper airways stimulated different inflammatory responses compared to the *H. parainfluenzae* isolated from lower airways on 16HBE cells

The four pairs of *H. parainfluenzae* from upper and lower airways (same strains as the last page) were then co-cultured with 16HBE cells for 8 hours at different bacterial load. CXCL8 produced from 16HBE cells were measured by ELISA. There was no significant difference in the level of CXCL8 produced from 16HBE cells when they were co-cultured with *H. parainfluenzae* from upper or lower airways at low bacterial load (10^3 cfu/mL to 10^6 cfu/mL). At high bacterial load (10^7 cfu/mL and 10^8 cfu/mL), *H. parainfluenzae* from upper airways induced significantly more CXCL8 production from 16HBE cells than the *H. parainfluenzae* from lower airways (p=0.017 and p=0.017 respectively; Figure 23). Median CXCL8 value for *H. parainfluenzae* from lower airways were: 644.7 pg/mL for 10^3 cfu/mL, 577.4 pg/mL for 10^4 cfu/mL, 647.9 pg/mL for 10^5 cfu/mL, 702.9 pg/mL for 10^6 cfu/mL, 1264.4 pg/mL for 10^7 cfu/m and 1917.0 pg/mL for 10^8 cfu/mL. Median CXCL8 value for *H. parainfluenzae* from upper airways were: 658 pg/mL for 10^3 cfu/mL, 611.4 pg/mL for 10^4 cfu/mL, 672.0 pg/mL for 10^5 cfu/mL, 802.5 pg/mL for 10^6 cfu/mL, 1805.2 pg/mL for 10^7 cfu/m and 2536.5 pg/mL for 10^8 cfu/mL (Figure 23).

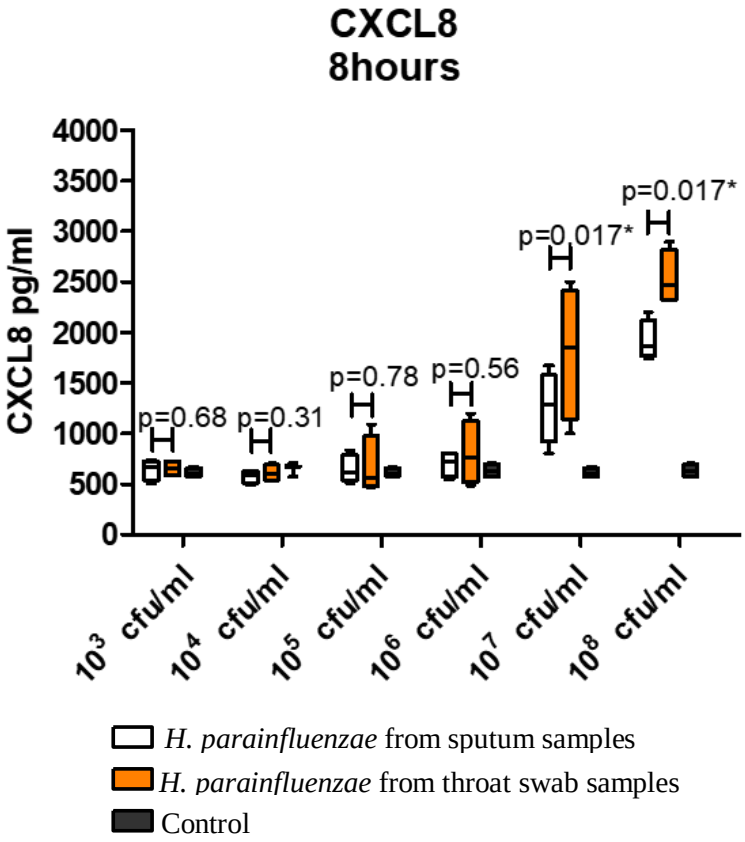


Figure 23. Inflammatory responses from 16HBE cells after co-cultured with *H. parainfluenzae* from patients' upper airways and lower airways. Four pairs of *H. parainfluenzae* from four individual patients' throat and sputum were co-cultured with 16HBE cells at different bacterial loads: 10^3 cfu/mL, 10^4 cfu/mL, 10^5 cfu/mL, 10^6 cfu/mL, 10^7 cfu/mL and 10^8 cfu/mL. Control group was same volume of PBS. After 8 hours, media was then collected. Media CXCL8 level was measured by ELISA. CXCL8 value was compared between *H. parainfluenzae* from sputum and throat swab. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8, data presented in box and whisker plots, the box shows +/- IQR, the line inside the box is median.

3.2.3 Conclusions for aim 1 and aim 2

- *Haemophilus* species are under-reported from traditional NHS microbiological laboratory methods, particularly with patients that had *H. parainfluenzae.*
- There were fewer patients that had found no dominant pathogen using our laboratory method which includes quantitative bacteriology and multiple molecular tests compared to the NHS microbiology laboratory results.
- In a limited sample size, the *H. parainfluenzae* isolates from upper airways and lower airways had a different but related genomic profile.
- Both *H. parainfluenzae* from lower and upper airways stimulated inflammatory responses at 10^7 cfu/mL and 10^8 cfu/mL. In a small sample size, *H. parainfluenzae* from upper airways stimulated greater inflammatory responses compared to lower airways' *H. parainfluenzae*.

3.3 Results for aim 3. Compare clinical features of *H. parainfluenzae* and NTHi in bronchiectasis patients

Having found that a relatively significant percentage of patients had *H. parainfluenzae*, and *H. parainfluenzae* was not reported from the NHS clinic at all, to figure out what kind of role *H. parainfluenzae* play in bronchiectasis lower airways became very crucial. This part of the study was mainly to investigate the sputum and serum inflammation of patients who had *H. parainfluenzae;* we compared those patients to the patients that had NTHi since NTHi have been regarded as pathogen in bronchiectasis.

Serum and sputum sol phase samples were collected from 63 patients that had either *H. parainfluenzae* or NTHi based on our laboratory method results. Those patients were all ≥18 years old, at a stable stage of bronchiectasis, and not on long term oral or inhaled antibiotics, no current smokers. Their Serum WCC, ESR, CRP and ICAM-1 were measured. Sputum sol phase MPO and sputum bacterial load were accessed. Patient's spirometry and BSI score were also analysed.

3.3.1 Patients clinical information

Patients were divided into two groups: 1) patients that had only *H. parainfluenzae* as the dominant potential pathogen in their sputum; 2) patients that had only NTHi as the dominant pathogen in their sputum. There were 24 patients had *H. parainfluenzae*, 39 patients had NTHi. Their clinical information was collected from the NHS. No significant difference was observed between these two groups of patients in their sex, age, smoking spirometry, the number of infected lobes and exacerbation in the past year (Table 13).

Table 13. Patient's information

	H. parainfluenzae	**NTHi**
Number	24	39
Sex F/M	12/12	22/17
Age	70 (21-82)	71 (23-93)
Number of ex- smoker (pack year)	9(11.5)	13 (12)
FEV_1 (L)	1.46 (0.6- 3.39)	1.81 (0.67- 3.87)
FEV_1% pred	69.1% (20.8%- 134.5%)	64.2% (21.85%- 161.25%)
FVC (L)	2.05 (1.27- 4.99)	2.76 (1.3- 6.3)
FVC% pred	78.4% (34.4%- 127.5%)	79.2% (48.2%- 138.25%)
Number of affected lobes	3 (2- 6)	3 (1- 6)
Median number exacerbation	1 (0- 6)	2 (0- 12)

Median value (min- max)
FEV_1% pred: FEV1% predicted
FVC% pred: FVC% predicted

3.3.2 The NTHi bacterial load in patients' samples was significantly higher than that of *H. parainfluenzae*

There was a significant difference between patients who had NTHi and *H. parainfluenzae* in their sputum bacterial load. The median bacterial load was 1.6×10^6 cfu/mL in the patients that had *H. parainfluenzae* and 1.06×10^8 cfu/mL in the patients with NTHi, $p<0.0001$ (Figure 24). Even though the bacterial load was lower in the patients that had *H. parainfluenzae*, there were 15 (60%) patients had more than 10^6 cfu/mL *H. parainfluenzae* in their sputum samples, which is a significant bacterial number in sputum samples in bronchiectasis.

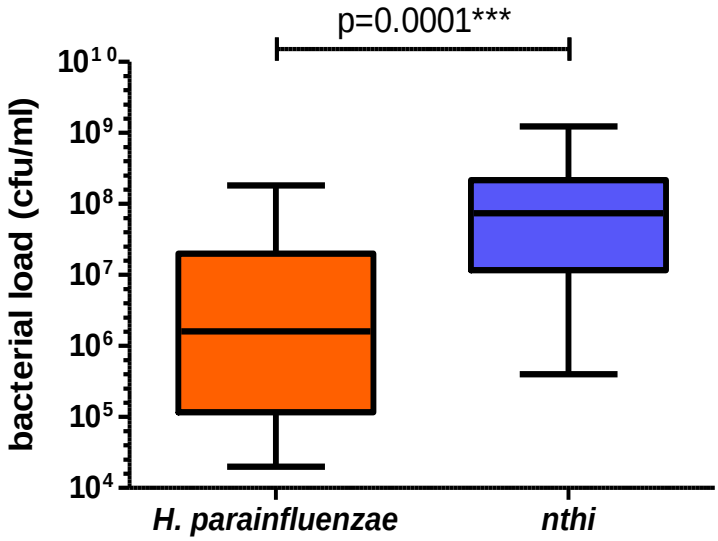


Figure 24. NTHi bacterial load and *H. parainfluenzae* load in patients. Patients sputum *H. parainfluenzae* (n=24) and NTHi (n=39) bacterial load was calculated and compared using unpaired t-test. Data are presented in min to max box and whisker plots. The box shows +/- IRQ. The line inside the box is median.

3.3.3 Patients had *H. parainfluenzae* and NTHi had similar serum WCC, ESR, CRP and ICAM-1 values

Patients' serum WCC, CRP, ESR and ICAM-1 values were measured. Patients that had *H. parainfluenzae*(n=24) had similar WCC, ESR, CRP and ICAM-1 as the patients that had NTHi (n=39). P-values are 0.06, 0.5, 0.62 and 0.52 (Figure 24). The median WCC, ESR, CRP and ICAM-1 were 6.9×10^9 /mL, 8.0 mm/h, 4.0 mg/L, 7903 pg/mL in patients that had *H. parainfluenzae*; 8.0×10^9 /mL, 8.0 mm/h, 4.0 mg/L, 7941 pg/mL in patients that had NTHi (Figure 25).

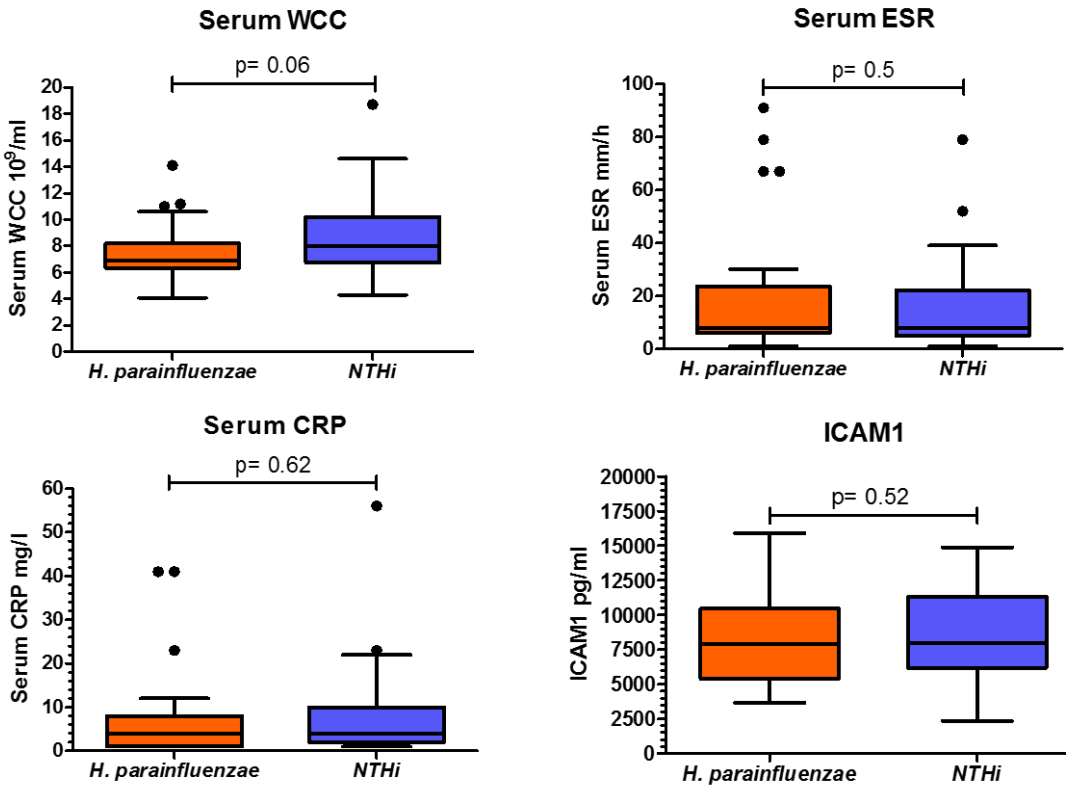


Figure 25. Serum WCC, ESR, CRP and ICAM level for patients that had *H. parainfluenzae* or NTHi. Patient's serum WCC, ESR, CRP and ICAM-1 were calculated and compared between patients that had *H. parainfluenzae* (n=24) and NTHi (n=39) in their sputum. Data are presented in Tukey box and whisker plots. The box shows +/- IQR. The line inside the box is median. The dots are outliers. P-values were calculated by unpaired t-test.

3.3.4 Patients that had higher sputum *H. parainfluenzae* bacterial load had higher serum ICAM-1 and ESR levels compared to the patients that had lower sputum *H. parainfluenzae* bacterial load

Those patients' serum WCC, CRP, ESR and ICAM-1 value were then compared between those had high sputum *H. parainfluenzae* bacterial load and those had low sputum *H. parainfluenzae* load. There were six patients in each group; their sputum bacterial load was higher or lower than the interquartile range (1.16×10^5 cfu/mL to 2.34×10^7 cfu/mL). The median bacterial load for low bacterial load group was 9×10^4 cfu/mL, for high bacterial load group was 3.4×10^7 cfu/mL. Serum ESR and ICAM-1 were significantly higher in patients that had high bacterial load (ESR=18 mm/h, ICAM-1=10446.7 pg/mL) compare to patients that had a lower bacterial load (ESR=6.5mm/h, p=0.04, ICAM-1=6616.7 pg/mL, p=0.02). There was no difference in serum WCC and CRP. (Figure 26)

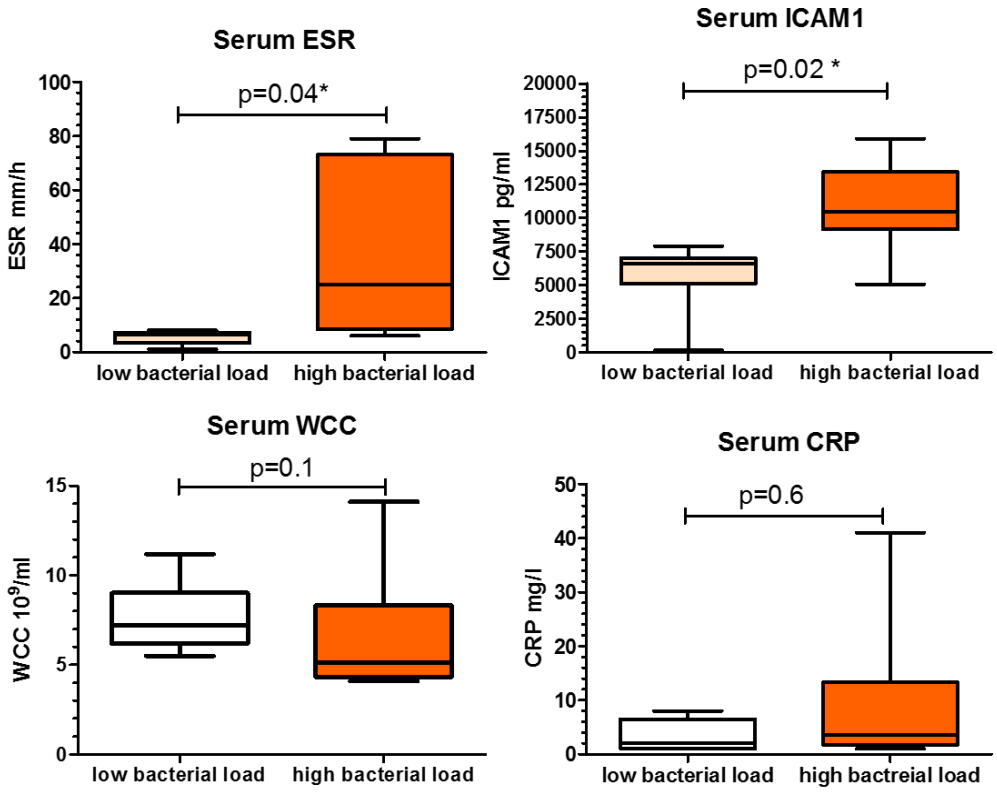


Figure 26. Serum WCC, ESR, CRP and ICAM-1 level of patients that had high bacterial load and low bacterial load. Patient's serum WCC, ESR, CRP and ICAM-1 were calculated and compared between patients that had high bacterial load (n=6) and low bacterial load (n=6) of *H. parainfluenzae* in their sputum. Those patients sputum bacterial load were either higher or lower than the interquartile range (1.16×10^5 cfu/mL to 2.34×10^7 cfu/mL). Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-values were calculated by unpaired t-test.

3.3.5 Patients that had NTHi or *H. parainfluenzae* had similar sputum MPO value

Patients' sputum MPO levels were measured. Similar to those serum inflammatory markers, there was no significant difference between patients who had NTHi (n=39) or *H. parainfluenzae* (n=24). The median MPO was 65.19 µg/mL in patients that had *H. parainfluenzae* (n=24), 116.4 µg/mL in patients that had NTHi (n=39), p=0.068 between groups (Figure 27).

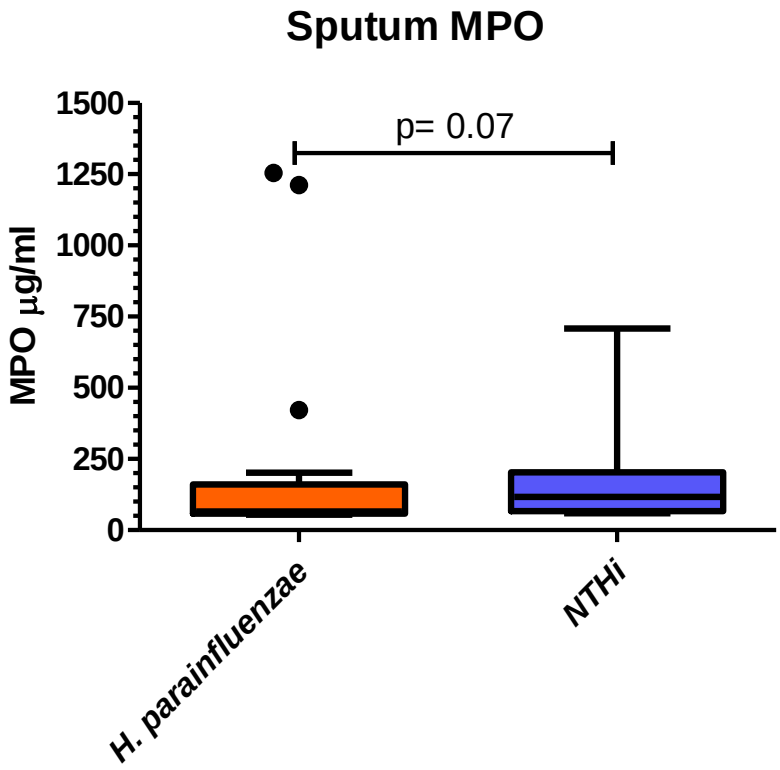


Figure 27. Sputum MPO level of the patients that had *H. parainfluenzae* or NTHi. Sputum MPO value of patient that had *H. parainfluenzae* (n=24) and NTHi (n=39), data are presented in Tukey box and whisker plots. The box shows +/- IQR. The line inside the box is median. The dots are outliers. P-value used unpaired t-test.

3.3.6 Patients had higher sputum *H. parainfluenzae* load had higher sputum MPO level

Sputum MPO values were compared between six patients who had high sputum *H. parainfluenzae* bacterial load (>2.34×10^7 cfu/mL) and six patients who had low sputum *H. parainfluenzae* bacterial load (<1.16×10^5 cfu/mL). The median bacterial load for low bacterial load group was 9×10^4 cfu/mL, for high bacterial load group was 3.4×10^7 cfu/mL. Sputum MPO was significantly higher in the patients that had a high bacterial load. Median MPO value for high bacterial load group was 311.7 µg/mL, for low bacterial load group was 57.4 µg/mL, p=0.0087 (Figure 28).

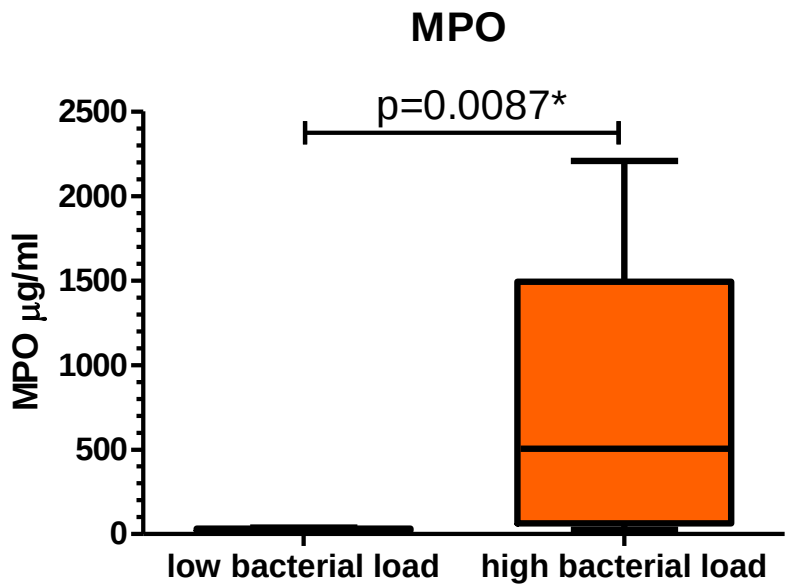


Figure 28. Sputum MPO of the patients that had high bacterial load and low bacterial load. Patient's sputum MPO was calculated and compared between patients that had higher load (n=6) and lower load (n=6) of *H. parainfluenzae* in their sputum. Those patients sputum bacterial load were higher than 2.34×10^7cfu/mL or lower than 1.16×10^5 cfu/mL. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value were calculated using an unpaired t-test.

3.3.7 Patients had NTHi or *H. parainfluenzae* had similar BSI scores

For their BSI score, there was no significant difference between patients who had NTHi (n=24) and *H. parainfluenzae* (n=39). The median BSI score was 7.0 in patients that had *H. parainfluenzae*, 7.0 in patients that had NTHi, p=0.9. (Figure 29a)

Those patients' BSI score was then compared between six patients who had high sputum *H. parainfluenzae* bacterial load (>2.34×10^7 cfu/mL) and six patients who had low sputum *H. parainfluenzae* bacterial load (<1.16×10^5 cfu/mL). The median bacterial load for low bacterial load group was 9×10^4 cfu/mL, for high bacterial load group was 3.4×10^7 cfu/mL. There was no significant difference in the BSI scores between these two groups. Median BSI score for high bacterial load group was 5.0, for low bacterial load group was 6.0, p=1.0. (Figure 29b)

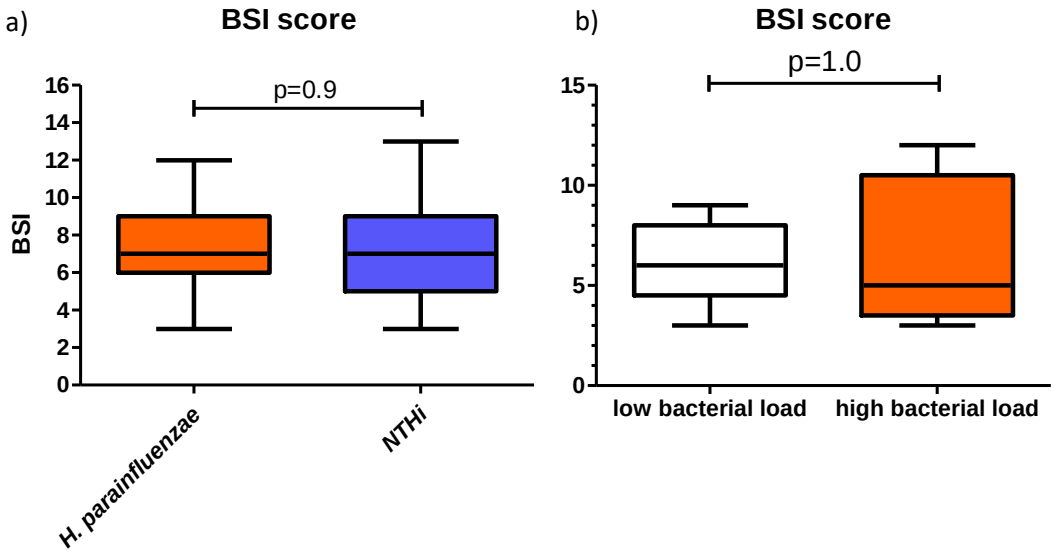


Figure 29. **Patients BSI scores**. a) BSI score for patients that had *H. parainfluenzae* (n=25) and NTHi (n=39). b) Patient's BSI score were calculated and compared between patients that had higher bacterial load (n=6) and lower bacterial load (n=6) of *H. parainfluenzae* in their sputum. Those patients sputum bacterial load were either higher or lower than the interquartile range (1.16×10^5 cfu/mL to 2.34×10^7 cfu/mL). Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value used unpaired t-test.

3.3.8 Conclusions for aim 3

- The bacterial load was lower in the patients that had *H. parainfluenzae* compared to the patients that had NTHi.
- Patients that had *H. parainfluenzae* or NTHi had similar sputum and serum inflammatory results.
- Patients that had *H. parainfluenzae* or NTHi had similar bronchiectasis severity, according to BSI scores.
- In a small sample size, in comparison of *H. parainfluenzae* in high bacterial load and low bacterial load, patients that had high *H. parainfluenzae* bacterial load had higher serum inflammation with raised ESR and ICAM-1, although no change in WCC and CRP. Sputum inflammation (MPO) was also higher in those patients.

3.4 Results for aim 4. Assess the impact of *H. parainfluenzae* on epithelial cells

From previously results, we have established that patients had *H. parainfluenzae* and NTHi were similar in their clinical features with similar serum inflammation and sputum inflammation. In addition, high bacterial load of *H. parainfluenzae* led to more inflammation in bronchiectasis. We wanted to establish further if *H. parainfluenzae* would trigger an inflammatory response as NTHi did *in vitro,* and if so, we wanted to see if the inflammatory responses were bacterial load related, subtype related, or culture time related. Hence, 12 unselected strains of *H. parainfluenzae,* NTHi and *P. aeruginosa* that were isolated from bronchiectasis patients were co-cultured with three different types of epithelial cells: human bronchial epithelial cell line (16HBE), normal human bronchial epithelial cells (NHBE) and bronchiectasis patients' primary nasal epithelial cells. We mainly looked into the cytokine production of cells after being co-cultured with bacteria.

3.4.1 16HBE CXCL8 production induced by *H. parainfluenzae* was culture time-dependent

After co-cultured with 16HBE cells for 2 hours, 10^7 cfu/mL *H. parainfluenzae* (n=6) induced significantly more CXCL8 production compared to the first hour, p=0.0001. p-value was calculated by paired t-test. There was a significant increase in CXCL8 level over 24 hours' co-culture time, p=0.0001. P-value was calculated by 1-way ANOVA. The production of CXCL8 reached to peak after 8 hours. There was no significant difference between 8 hours and 24 hours. The median CXCL8 fold change compared to negative control was 0.9 for 1 hour, 1.9 for 2 hours, 4.1 for 4 hours, 3.9 for 6 hours, 6.9 for 8 hours and 5.1 for 24 hours (Figure 30).

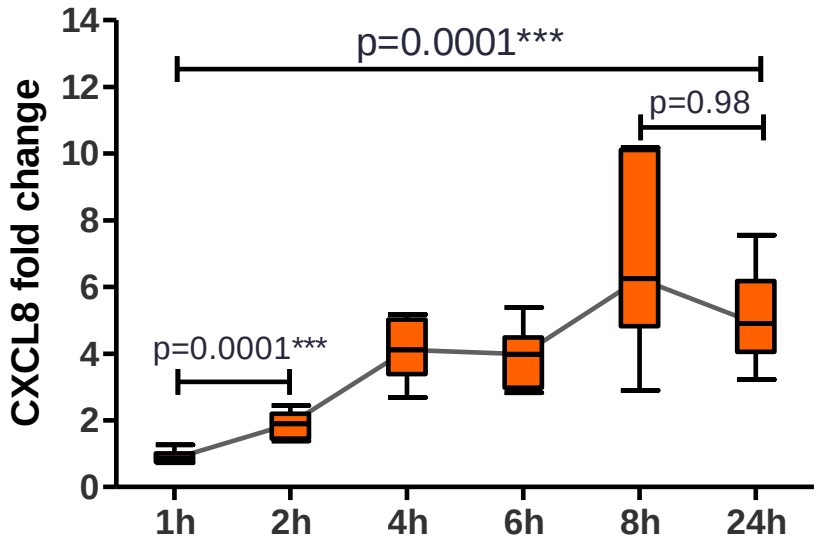


Figure 30. CXCL8 production after 16HBE cells co-cultured with *H. parainfluenzae*. 16HBE cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae* (n=6) for 1 hour, 2 hours, 4 hours, 6 hours, 8 hours and 24 hours, control was same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 compare to average negative control. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was 1 way ANOVA.

3.4.2 16HBE CXCL8 production induced by NTHi was culture time related

After co-cultured with 16HBE cells for 2 hours, 10^7 cfu/mL NTHi (n=6) induced significantly more CXCL8 production compared to the first hour, p=0.0001. P-value was calculated by paired t-test. There was no significant difference between 8 hours and 24 hours. There was a significant increase in CXCL8 level over the 24 hours' co-culture time, p=0.0001. P-value was calculated by 1-way ANOVA. The median CXCL8 fold change compared to negative control was 1.2 for 1 hour, 2.4 for 2 hours, 4.3 for 4 hours, 3.3 for 6 hours, 4.6 for 8 hours and 4.0 for 24 hours (Figure 31).

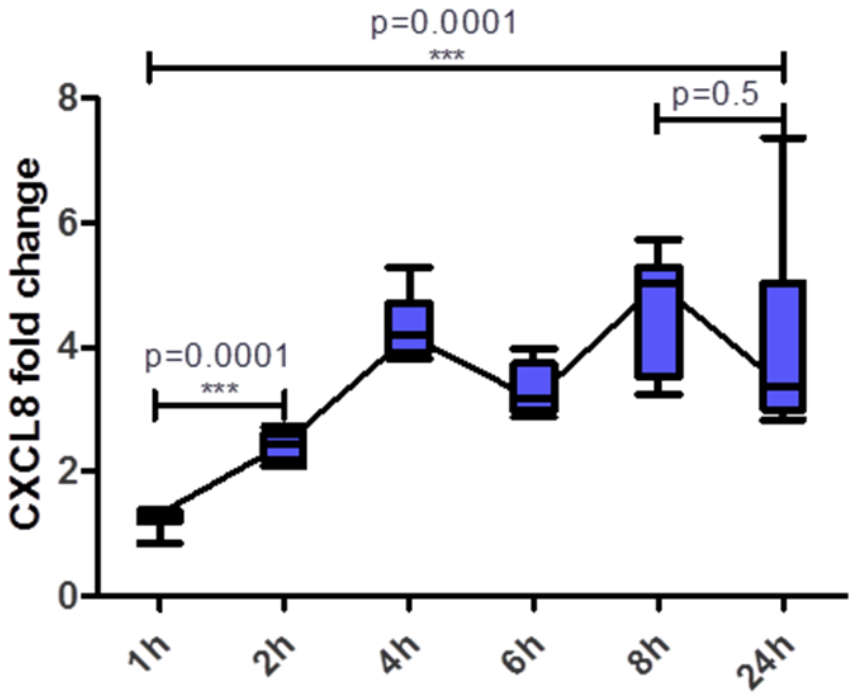


Figure 31. CXCL8 production after 16HBE cells co-cultured with NTHi. 16HBE cells were co-cultured with 10^7 cfu/mL NTHi (n=6) for 1 hour, 2 hours, 4 hours, 6 hours, 8 hours and 24 hours, control was same volume of PBS. 100µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 compare to negative control. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was 1 way ANOVA.

3.4.3 16HBE CXCL8 production induced by *P. aeruginosa* was culture time-dependent

After co-cultured with 16HBE cells, 10^7 cfu/mL *P. aeruginosa* (n=6) induced significantly more CXCL8 production after 2 hours, p=0.007. Different from *H. parainfluenzae* and NTHi, there was significant higher CXCL8 level at 24 hours compared to 8 hours. The production of CXCL8 was positively correlated with culture time, p=0.0001. P-value was calculated by 1-way ANOVA. The median CXCL8 fold change compared to negative control was 0.9 for 1 hour, 1.8 for 2 hours, 6.8 for 4 hours, 6.6 for 6 hours, 6.8 for 8 hours and 8.7 for 24 hours (Figure 32).

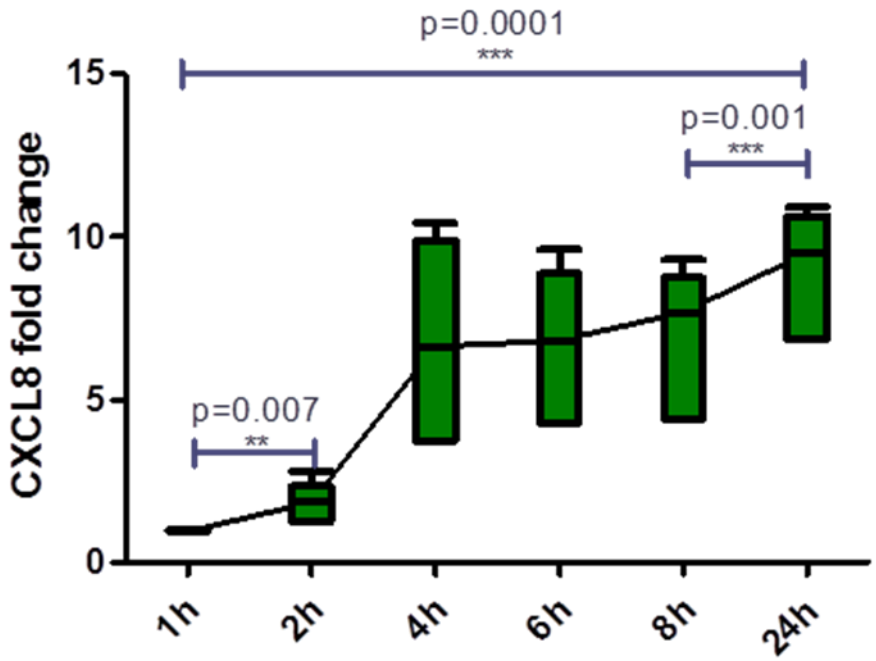


Figure 32. CXCL8 production after 16HBE cells co-cultured with *P. aeruginosa*. 16HBE cells were co-cultured with 10^7 cfu/mL *P. aeruginosa* (n=6) for 1 hour, 2 hours, 4 hours, 6 hours, 8 hours and 24 hours, control was same volume of PBS. 100 μL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 compare to negative control. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was 1 way ANOVA.

3.4.4 *P. aeruginosa* stimulated significantly more CXCL8 production than *H. parainfluenzae* and NTHi at 24 hours

After co-cultured with 16HBE cells for 8 hours, the CXCL8 level from both 10^7 cfu/mL *H. parainfluenzae* (n=6) and 10^7 cfu/mL NTHi (n=6) groups reached to peak. The 16HBE cells that were co-cultured with 10^7 cfu/mL *P. aeruginosa* (n=6), however, produced more CXCL8 at 24 hours than at 8 hours.

At 24 hours, *P. aeruginosa* triggered significantly more CXCL8 production than *H. parainfluenzae* and NTHi (p=0.02 and p=0.006 respectively). P-value was calculated by unpaired t-test. The median CXCL8 value for *H. parainfluenzae* was 1571 pg/mL, for NTHi was 1082 pg/mL and for *P. aeruginosa* was 3048 pg/mL (Figure 33).

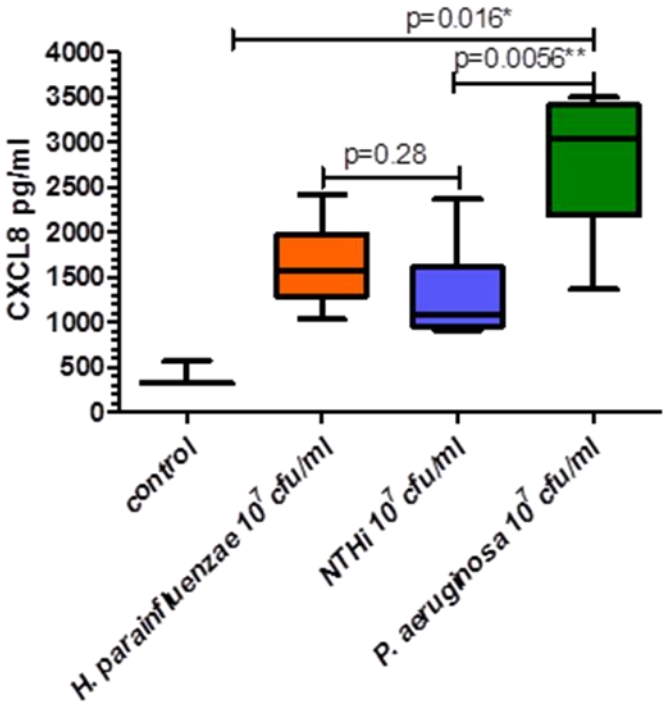


Figure 33. CXCL8 production after 16HBE cells co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* for 24 hours. 16HBE cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae* (n=6), 10^7 cfu/mL NTHi (n=6) or 10^7 cfu/mL *P. aeruginosa* (n=6) for 24 hours, control was same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data is presented in min to max box and whisker plots. The box shows +/- IRQ. The line inside the box is median. Y axis shows CXCL8 value. P-values were calculated by unpaired t-test.

3.4.5 *H. parainfluenzae* significantly stimulated cells LDH releasement after 24 hours of culture time

After 8 hours, the production of CXCL8 from 16HBE cells caused by *H. parainfluenzae* reached to the peak. We further checked the cells LDH releasement at 8 hours and 24 hours.

After 16HBE cell has been co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* (10^7 cfu/mL, n=6) for 8 hours, there was no significant LDH release compared to the control group. The median LDH release for the control group was 6%, for *H. parainfluenzae* was 9%, for NTHi was 8% and for *P. aeruginosa* was 10%. After 24 hours, 16HBE cells that were co-cultured with bacteria had significantly higher LDH level compared to the control group (*H. parainfluenzae* p=0.016, NTHI p=0.02, *P. aeruginosa* p=0.016). There was no significant difference between *H. parainfluenzae*, NTHi and *P. aeruginosa* groups at both 8 hours and 24 hours. The median LDH release at 24 hours for control was 6.5%, for *H. parainfluenzae* was 29%, for NTHi was 24% and for *P. aeruginosa* was 43%. Because of the low rate of LDH release at 8 hours, 8 hours was chosen to be the time point for further cell-bacteria co-culture experiments (Figure 34).

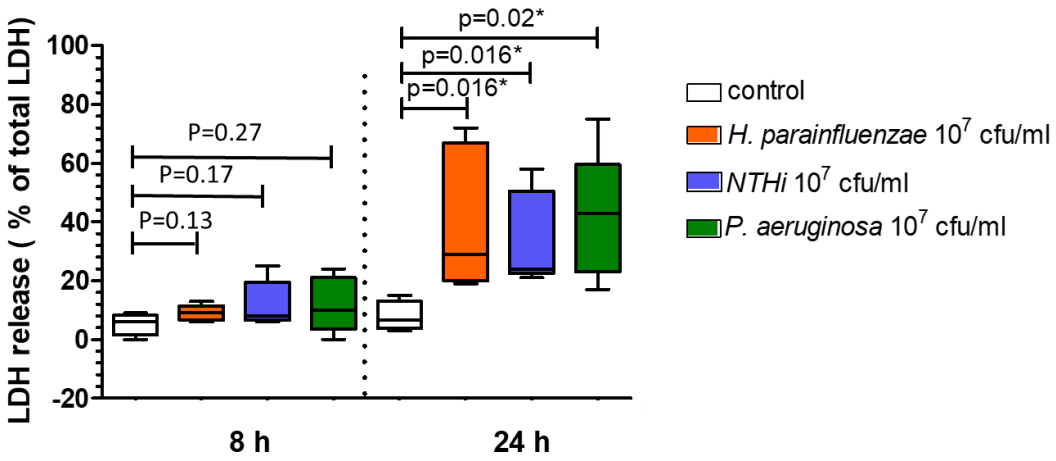


Figure 34. LDH release from 16HBE cells after co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* for 8 hours and 24 hours. 16HBE cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae* (n=6), 10^7 cfu/mL NTHi (n=6), 10^7 cfu/mL *P. aeruginosa* (n=6) for 8 hours and 24 hours, control was same volume of PBS. 100 µL of cell culture supernatant was run on each LDH assay. Data is presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows LDH release compared to the total LDH which was calculated based on the LDH assay results. P-values was calculated by non- paired t-test.

3.4.6 16HBE cells inflammatory responses were dose-dependent

From the previous patient's serum ESR, WCC and sputum MPO results, we found that higher bacterial load was related to higher inflammatory responses. So, we proposed that *H. parainfluenzae*'s inflammatory effect on 16HBE cells was bacterial load-dependent. *H. parainfluenzae* (n=6), NTHi (n=6) and *P. aeruginosa* (n=6) at 10^4 cfu/mL, 10^5 cfu/mL, 10^6 cfu/mL, 10^7 cfu/mL and 10^8 cfu/mL were cultured with 16HBE cells for 8 hours.

For *H. parainfluenzae*, at 10^4 cfu/mL, 10^5 cfu/mL and 10^6 cfu/mL, there was no significant CXCL8 production compared to the control group. At 10^7 cfu/mL and 10^8 cfu/mL, the CXCL8 production significantly increased compared to the control group (p=0.03 and p=0.004 respectively). Using 1-way ANOVA test shows there was a significant overall increase in CXCL8 production (p=0.0001). The median CXCL8 value for 10^4 cfu/mL *H. parainfluenzae* group was 272.5 pg/mL, for 10^5 cfu/mL group was 275 pg/mL, for 10^6 cfu/mL group was 325 pg/mL, for 10^7 cfu/mL group was 417 pg/mL and 10^8 cfu/mL group were 944.6 pg/mL (Figure 35a).

Similar to *H. parainfluenzae*, NTHi induced significant CXCL8 production at 10^7 cfu/mL and 10^8 cfu/mL (p=0.02 and p=0.035 respectively). 1-way ANOVA test shows there was a significant overall increase in CXCL8 production (p=0.0002). The median CXCL8 value for 10^4 cfu/mL NTHi group was 334.2 pg/mL, for 10^5 cfu/mL group was 311.2 pg/mL, for 10^6 cfu/mL group was 366 pg/mL, for 10^7 cfu/mL group was 516.7 pg/mL and 10^8 cfu/mL group were 881 pg/mL (Figure 35b).

P. aeruginosa was different from the two *Haemophilus* species, from 10^5 cfu/mL, *P. aeruginosa* already stimulated significant more CXCL8 production (p=0.02) compared to the control group. Similar to *H. parainfluenzae* and NTHi, higher bacterial load of *P. aeruginosa* can trigger higher CXCL8 production. 1-way ANOVA test shows there was a significant overall increase in CXCL8 production (p=0.0001). The median CXCL8 value for 10^4 cfu/mL *P. aeruginosa* group was 288.6 pg/mL, for 10^5 cfu/mL group was 497.1 pg/mL, for 10^6 cfu/mL group was 588.2 pg/mL, for 10^7 cfu/mL group was 1000 pg/mL and 10^8 cfu/mL group 1310 pg/mL (Figure 35c).

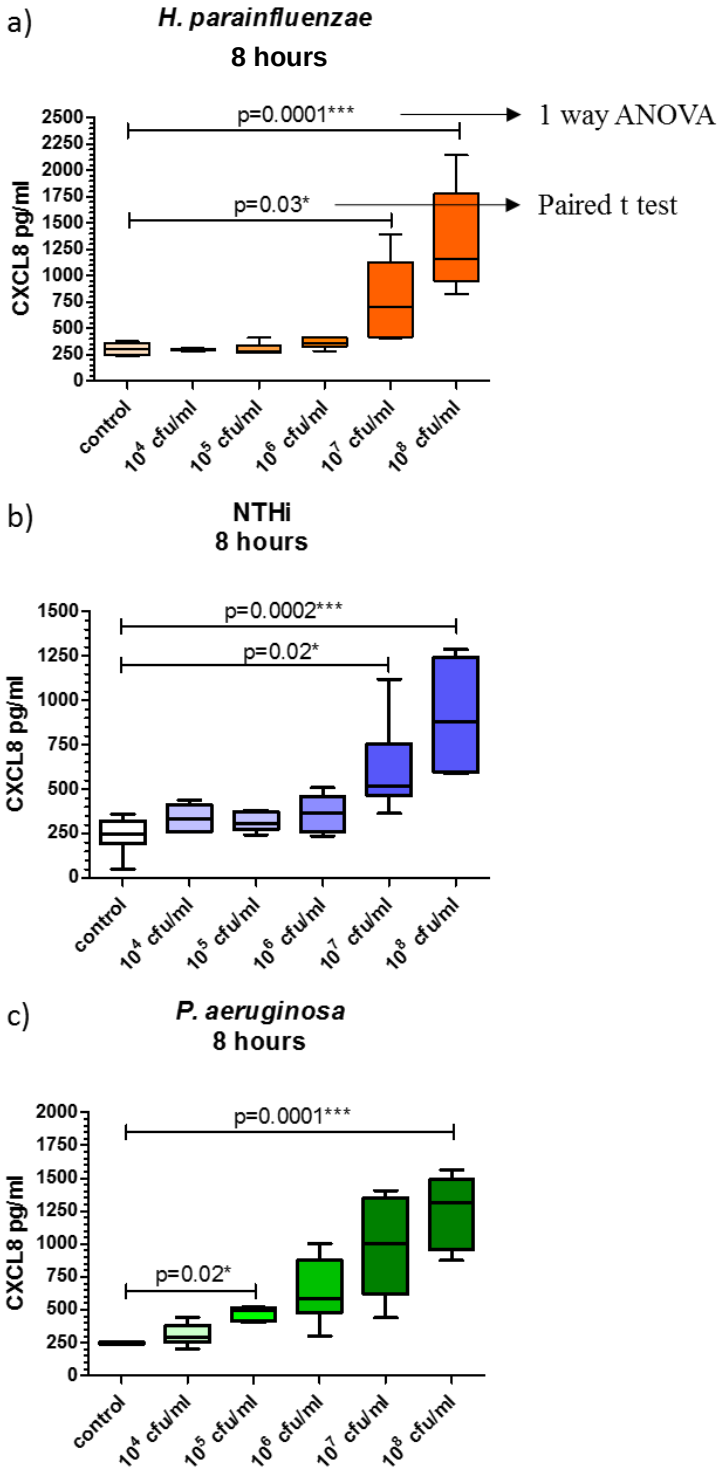
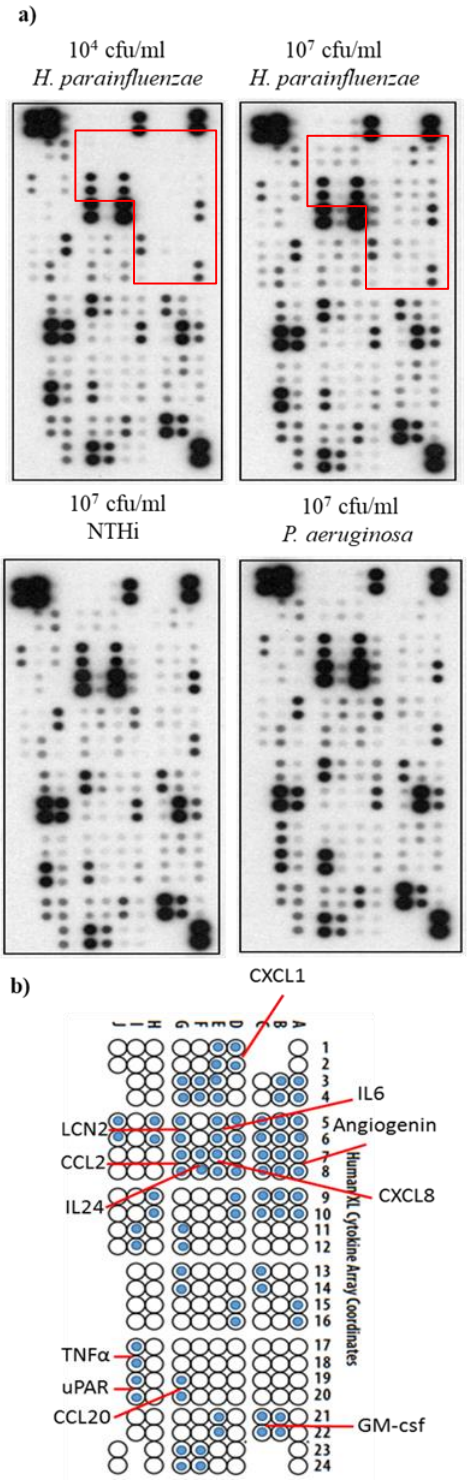


Figure 35. CXCL8 production of 16HBE cells after co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* for 8 hours. 16HBE cells were co-cultured with 10^4 cfu/mL, 10^5 cfu/mL, 10^6 cfu/mL, 10^7 cfu/mL and 10^8 cfu/mL *H. parainfluenzae* (n=6), *H. parainfluenzae* (n=6), NTHi (n=6) and *P. aeruginosa* (n=6) for 8 hours. Control was same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data is presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of CXCL8. P-values were calculated by paired t-test and 1 way ANOVA.

3.4.7 *H. parainfluenzae* can cause a similar range of inflammatory responses on 16HBE cell line as *P. aeruginosa*

From previous experiments, we have found that after 8 hours and at 10^7 cfu/mL, *H. parainfluenzae*, NTHi and *P. aeruginosa* can stimulate significant CXCL8 production, and we wanted to detect whether *H. parainfluenzae* would also stimulate a broad inflammatory response from 16HBE cells. 105 cytokines were measured after 16HBE cells had been co-cultured with 10^4 cfu/mL of *H. parainfluenzae* (n=6), 10^7 cfu/mL of *H. parainfluenzae* (n=6), 10^7 cfu/mL of NTHi (n=6) or 10^7 cfu/mL of *P. aeruginosa* (n=6) for 8 hours using a cytokine antibody microarray (Figure 36 and Figure 37). These bacteria were all isolated from our bronchiectasis patients' sputum samples. The cytokine production levels were expressed as spots in the results of the microarray. The average expression levels of spot density stimulated by the four bacterial groups for 42 of these cytokines are shown in figure 37. There was a visible difference in the cytokine profile between 16HBE cells co-cultured with *H. parainfluenzae* at a concentration of 10^4 cfu/mL and those cultured at 10^7 cfu/mL. We then detected and semi-quantified 18 cytokines with higher production levels in the 10^7 cfu/mL groups compared to the 10^4 cfu/mL group: Angiopoietin 1 (ANG1), basic fibroblast growth factor (FGF-2), Hepatocyte growth factor (HGF), Leukemia inhibitory factor (LIF), angiogenin, CD40 ligand, Chemokine ligand 20 (CCL20), IL6, TNFα, CXCL1, Granulocyte-macrophage colony-stimulating factor (GM-csf), CXCL8, LCN2, Urokinase receptor (uPAR), Interleukin 24 (IL24) and ICAM-1. There was no difference between cells co-cultured at 10^7 cfu/mL of *H. parainfluenzae*, NTHi or *P. aeruginosa*, which suggests that *H. parainfluenzae* induced similar cell inflammatory responses as NTHi and *P. aeruginosa*. The same experiment has been performed twice, resulting in different signal levels, but similar trend between bacterial groups.



c)

A3,4	Adiponectin
A5,6	Apolipoprotein A-I
A7,8	Angiogenin
A9,10	Angiopoietin-1
A15,16	BDNF
B3,4	CD40L
B5,6	CHI3L1
B7,8	Complement Factor D
B9,10	CRP
B21,22	Emmprin
C5,6	Endoglin
C7,8	Fas Ligand
C9,10	FGF-2
C13,14	FGF-19
C21,22	GM-CSF
D1,2	CXCL1
D5,6	HGF
D7,8	ICAM-1
D9,10	IFN-γ
D15,16	IL-1α
E1,2	IL-4
E3,4	IL-5
E5,6	IL-6
E7,8	IL-8
E21,22	IL-17A
F3,4	IL-22
F7,8	IL-24
F23,24	Kallikrein 3
G3,4	LIF
G5,6	Lipocalin-2
G7,8	MCP-1
G11,12	M-CSF
G13,14	MIF
G19,20	CCL20
G23,24	MMP-9
H5,6	PDGF-AA
H9,10	Pentraxin 3
I11,12	TfR
I17,18	TNF-α
I19,20	uPAR
J5,6	Vitamin D BP

Figure 36. Cytokine array results. 16HBE cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=6), 10^7 cfu/mL *H. parainfluenzae* (n=6), 10^7 cfu/mL NTHi (n=6) and 10^7 cfu/mL *P. aeruginosa* (n=6) for 8 hours. 600 µL of pooled cell culture supernatant was run on each array. Cell culture experiment was repeated and the second array was performed in the same manner. a) Shows the membranes of those four groups from one array assay. Those membranes were exposed to X-ray for 3 minutes. b) Blue marks represent the cytokines that expressed in higher levels in high bacterial load cell culture groups. c) Cytokine name and row number.

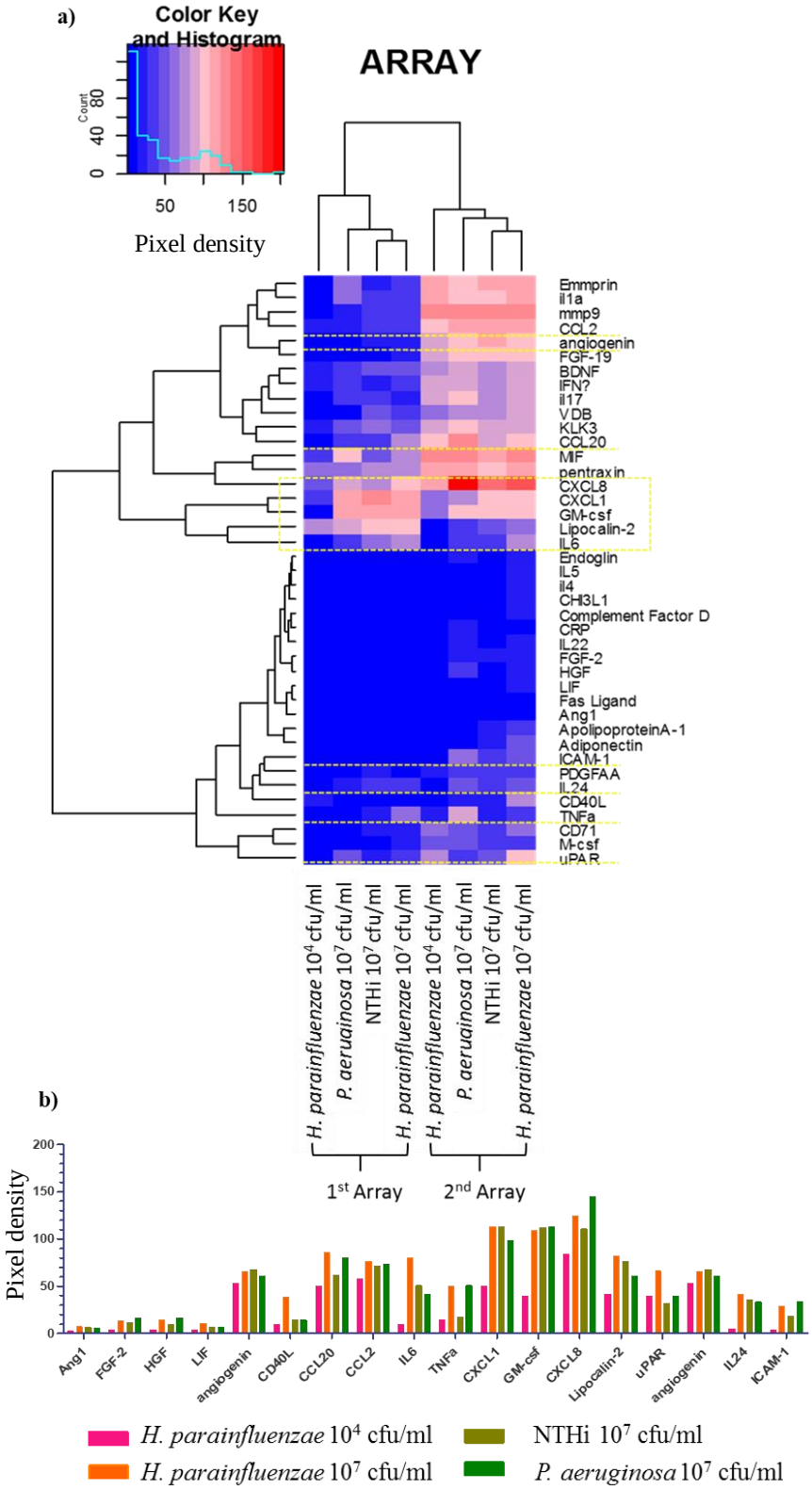


Figure 37. Pixel density of the cytokines measured in the cytokine array. a) Shows the pixel density of the blue colour marked cytokines from two separate cytokine array experiments. Yellow lines highlighted our interested cytokines. Pixel density were calculated by Image J. Heat map were generated using R. b) Average pixel density of two arrays for those 18 cytokines.

3.4.8 *H. parainfluenzae* induced CXCL8 production by 16 HBE cells at 8 hours in a similar manner as NTHi and *P. aeruginosa*

CXCL8 was measured and quantified by ELISA after previous cytokine array. 12 different clinical strains of *H. parainfluenzae*, NTHi and *P. aeruginosa* were co-cultured with 16HBE cells for 8 hours. A low bacterial load (10^4 cfu/mL) of *H. parainfluenzae* did not induce a significant CXCL8 production compared to the control group. A high bacterial load (10^7 cfu/mL) of *H. parainfluenzae* (n=12) induced a significant higher CXCL8 production when co-cultured with 16HBE cells compared to the 10^4 cfu/mL group and the control group. The median CXCL8 value for the 10^7 cfu/mL *H. parainfluenzae* group was 1711 pg/mL, and for the 10^4 cfu/mL *H. parainfluenzae* group it was 766.4 pg/mL, p=0.0016. P-value was calculated by paired t-test. NTHi (n=12) and *P. aeruginosa* (n=12) at 10^7 cfu/mL induced significantly more CXCL8 production compared to the control group (p=0.002 and p=0.0004 respectively). There was no significant difference between the 10^7 cfu/mL *H. parainfluenzae* (1711 pg/mL), 10^7 cfu/mL NTHi (1267 pg/mL) and 10^7 cfu/mL *P. aeruginosa* (1881 pg/mL) groups. P-value was calculated by unpaired t-test (Figure 38).

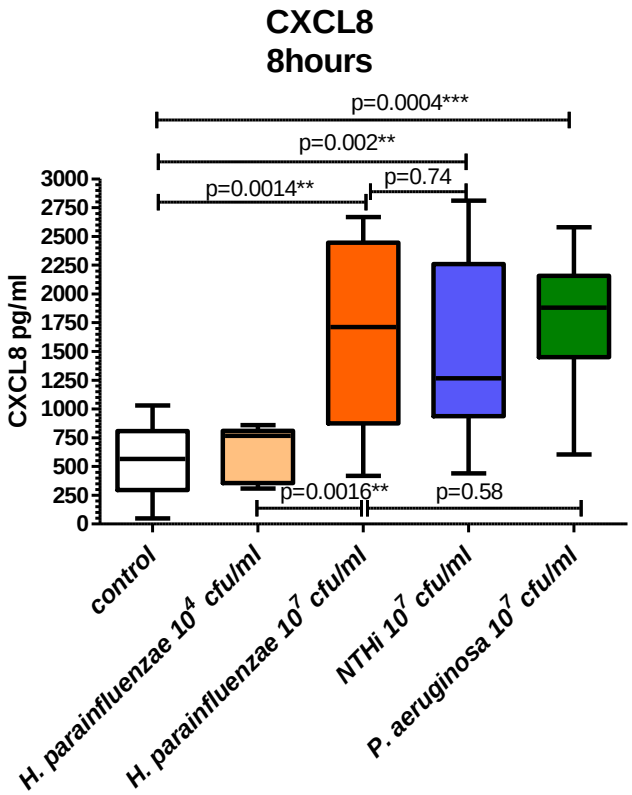


Figure 38. CXCL8 production from 16HBE cells after co-cultured with 10^7 cfu/ml *H. parainfluenzae*, NTHi and *P. aeruginosa*. 16HBE cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL NTHi (n=12) and 10^7 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of CXCL8. P-value between 10^4 cfu/mL *H. parainfluenzae* and 10^7 cfu/mL *H. parainfluenzae* was calculated by paired t-test. The rest of p-values were calculated by unpaired t-test.

3.4.9 *H. parainfluenzae* induced CXCL1 production by 16 HBE cells at 8 hours in a similar manner as NTHi and *P. aeruginosa*

Similar to CXCL8, 10^4 cfu/mL *H. parainfluenzae* did not induce significant CXCL1 production compared to the control group. High bacterial load (10^7 cfu/mL) of *H. parainfluenzae* (n=12) induced significant higher CXCL1 from 16HBE cells compared to 10^4 cfu/mL *H. parainfluenzae*. Median CXCL1 value for 10^7 cfu/mL *H. parainfluenzae* group was 986.3 pg/mL, for 10^4 cfu/mL *H. parainfluenzae* group was 176.4 pg/mL. P-value (p=0.003) was calculated by paired t-test.

NTHi (n=12) and *P. aeruginosa* (n=12) at 10^7 cfu/mL induced significantly more CXCL1 production than the control group (p=0.01 and p=0.001 respectively). There was no significant difference between 10^7 cfu/mL *H. parainfluenzae* (986.3 pg/mL), NTHi (813.2 pg/mL) and *P. aeruginosa* (1092 pg/mL). P-value was calculated by unpaired t-test. (Figure 39)

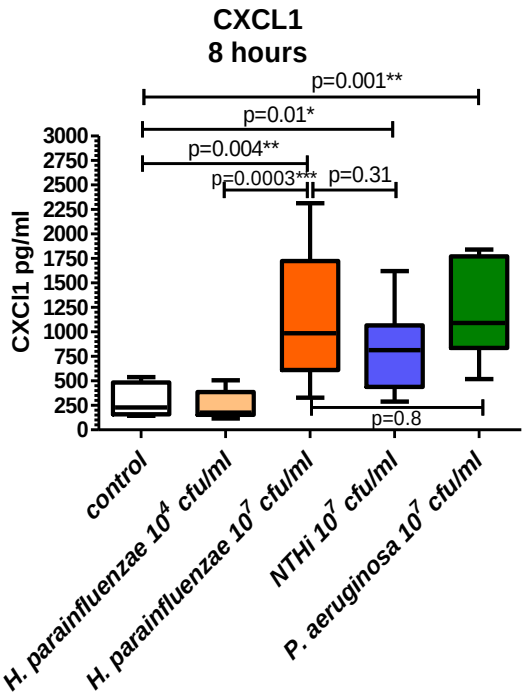


Figure 39. CXCL1 production from 16HBE cells after co-cultured with 10^7 cfu/ml *H. parainfluenzae*, NTHi and *P. aeruginosa*. 16HBE cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL NTHi (n=12) and 10^7 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of CXCL1. P-value between 10^4 cfu/mL *H. parainfluenzae* and 10^7 cfu/mL *H. parainfluenzae* was calculated by paired t-test. The rest of p-values were calculated by unpaired t-test.

3.4.10 *H. parainfluenzae* induced LCN2 production by 16 HBE cells at 8 hours in a similar manner as NTHi and *P. aeruginosa*

10^4 cfu/mL *H. parainfluenzae* did not induce significant LCN2 production compared to the control group. High bacterial load (10^7 cfu/mL) of *H. parainfluenzae* (n=12) induced significant more LCN2 when co-cultured with 16HBE cells compared to 10^4 cfu/mL. Median LCN2 value for 10^7 cfu/mL *H. parainfluenzae* group was 76.7 pg/mL, for 10^4 cfu/mL *H. parainfluenzae* group was 65.6 pg/mL. P-value (p=0.01) was calculated by paired t-test.

P. aeruginosa (n=12) at 10^7 cfu/mL can induce significant LCN2 production compared to the control group (p=0.017). There was no significant difference between 10^7 cfu/mL *H. parainfluenzae* (76.7 pg/mL), NTHi (151.7 pg/mL) and *P. aeruginosa* (371.1 pg/mL). P-value was calculated by unpaired t-test. (Figure 40)

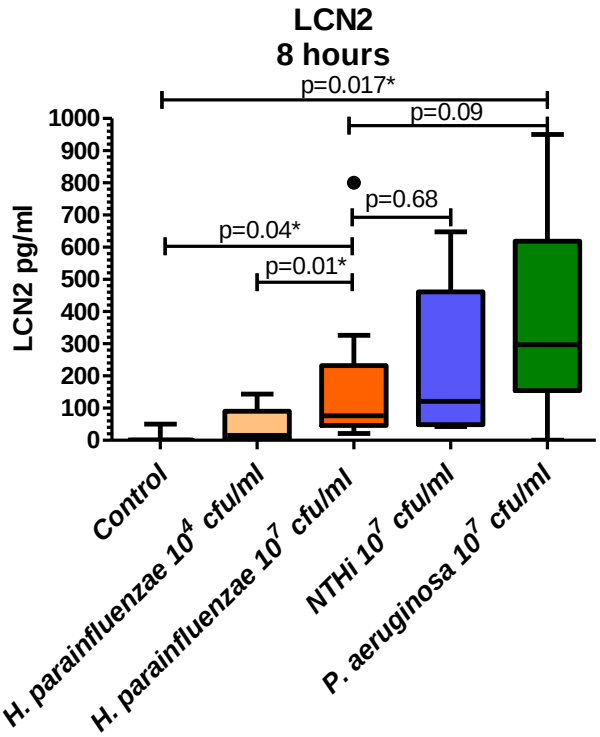


Figure 40. LCN2 production from 16HBE cells after co-cultured with 10^7 cfu/ml *H. parainfluenzae*, NTHi and *P. aeruginosa*. 16HBE cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL NTHi (n=12) and 10^7 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was co-cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of LCN2. P-value between 10^4 cfu/mL *H. parainfluenzae* and 10^7 cfu/mL *H. parainfluenzae* was calculated by paired t-test. The rest of p-values were calculated by unpaired t-test.

3.4.11 *H. parainfluenzae* induced ICAM-1 production from 16 HBE cells at 8 hours in a similar manner as NTHi and *P. aeruginosa*

10^4 cfu/mL *H. parainfluenzae* did not induce significant ICAM-1 production to compared to the control group. 10^7 cfu/mL *H. parainfluenzae* (n=12) induced significant more ICAM-1 when co-cultured with 16HBE cells compared to 10^4 cfu/mL. Median ICAM-1 value for 10^7 cfu/mL *H. parainfluenzae* group was 64.7 pg/mL, for 10^4 cfu/mL *H. parainfluenzae* group was 42.3 pg/mL. P-value (p=0.002) was calculated by paired t-test.

NTHi (n=12) and *P. aeruginosa* (n=12) in 10^7 cfu/mL induced significantly more ICAM-1 production than the control group (p=0.02 and p=0.006 respectively). There was no significant difference between 10^7 cfu/mL *H. parainfluenzae* (64.7 pg/mL), NTHi (54.1 pg/mL) and *P. aeruginosa* (56.3 pg/mL). P-value was calculated by unpaired t-test. (Figure 41)

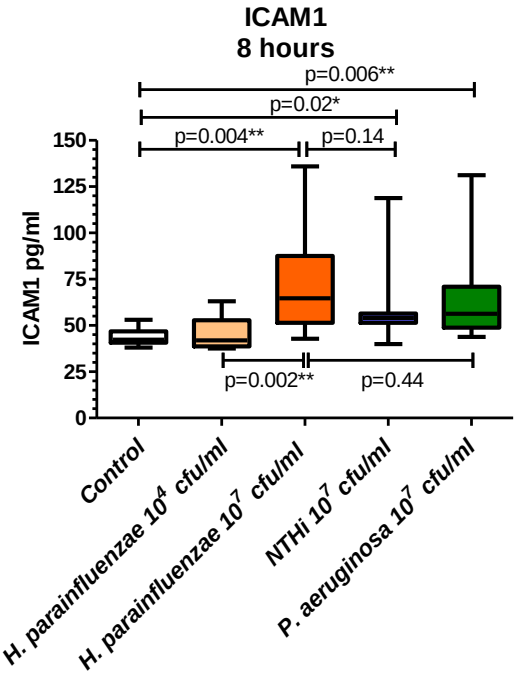


Figure 41. ICAM-1 production from 16HBE cells after co-cultured with 10^7 cfu/ml *H. parainfluenzae*, NTHi and *P. aeruginosa*. 16HBE cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL *H. parainfluenzae* (n=12), 10^7 cfu/mL NTHi (n=12) and 10^7 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of ICAM-1. P-value between 10^4 cfu/mL *H. parainfluenzae* and 10^7 cfu/mL *H. parainfluenzae* was calculated by paired t-test. The rest of p-values were calculated by unpaired t-test.

3.4.12 16HBE cell did not produce significant level of IL1β

Cytokine array results showed there was no production of IL1β from16HBE cells in both bacteria groups: *H. parainfluenzae* at 10^7 cfu/mL, NTHi at 10^7 cfu/mL and *P. aeruginosa* at10^7 cfu/mL. We confirmed this result using the ELISA test.

There was no detectable production of IL1β when 16HBE cells were cultured with PBS or with 10^5 cfu/mL to 10^7 cfu/mL of *H. parainfluenzae* (n=6). When cells co-cultured with 10^8 cfu/mL *H. parainfluenzae*, there was increased production of IL1β, but iss not statistically significant compared to the control group.

When 16HBE cells were cultured with a different bacterial load of NTHi (n=6), there was no IL1β detected by ELISA (Figure 42a).

Similar to *H. parainfluenzae*, when 16HBE cells co-cultured with *P. aeruginosa* (n=6), there was no detectable IL1β production at control, 10^5 cfu/mL, 10^6 cfu/mL and 10^7 cfu/mL groups. There was very low and not significant production of IL1β at 10^8 cfu/mL *P. aeruginosa* group (Figure 42b and c).

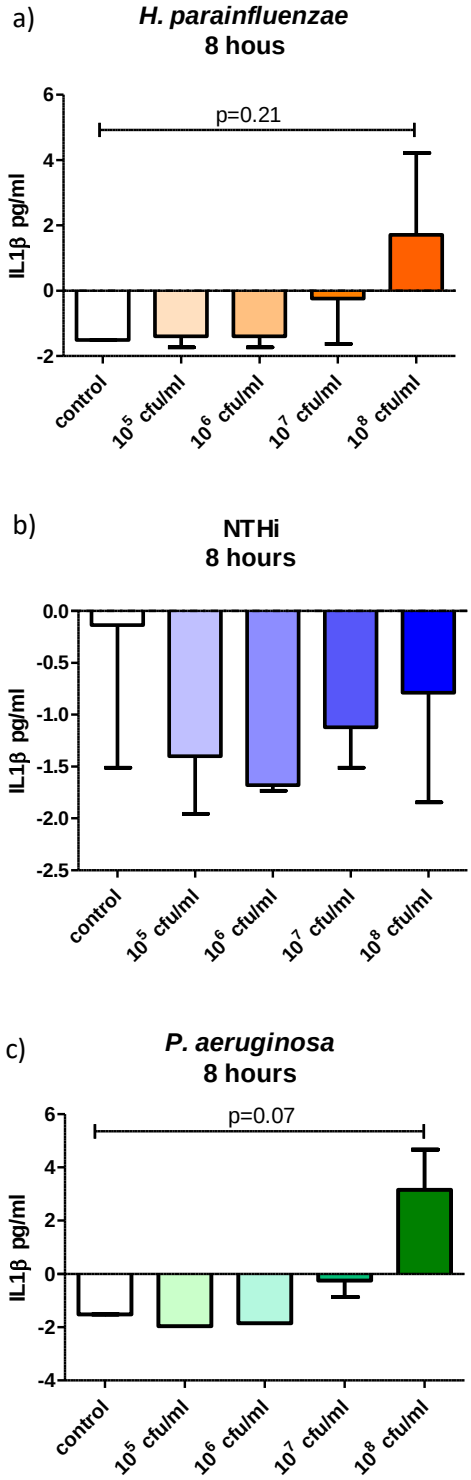


Figure 42. IL1 β production from 16HBE cells after co-cultured with 10^7 cfu/ml *H. parainfluenzae*, NTHi and *P. aeruginosa*.16HBE cells were co-cultured with 10^5 cfu/mL, 10^6 cfu/mL, 10^7 cfu/mL and 10^8 cfu/mL *H. parainfluenzae* (n=6), *H. parainfluenzae* (n=6), NTHi (n=6) and *P. aeruginosa* (n=6) for 8 hours. Control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of IL1 β . P-values were calculated by unpaired t-test.

3.4.13 Different subtypes of *H. parainfluenzae* induced similar inflammatory effects on 16HBE cells

In bronchiectasis patients, various *H. parainfluenzae* subtypes were isolated from patients' sputum samples. We wanted to elucidate whether different subtypes of *H. parainfluenzae* would have different inflammatory effects on 16HBE cells.

There was no significant difference in CXCL1 and CXCL8 production between different *H. parainfluenzae* subtypes. P-value was calculated using 1-way ANOVA. However, only one strain of type IV *H. parainfluenzae* and two strains of type VII *H. parainfluenzae* were identified; a higher availability of strains may change the P-value. The median CXCL8 fold change compared to the control group for subtype I was 1.42, for subtype II, 1.5; for subtype III, 1.9; subtype IV, 1.14; subtype V, 1.34, and for subtype VII, 1.1. The median CXCL1 fold change for subtype I was 1.374; for subtype II, 1.429; for subtype III, 1.633; for subtype IV, 1.077; for subtype V, 2.848, and for subtype VII, 2.524.

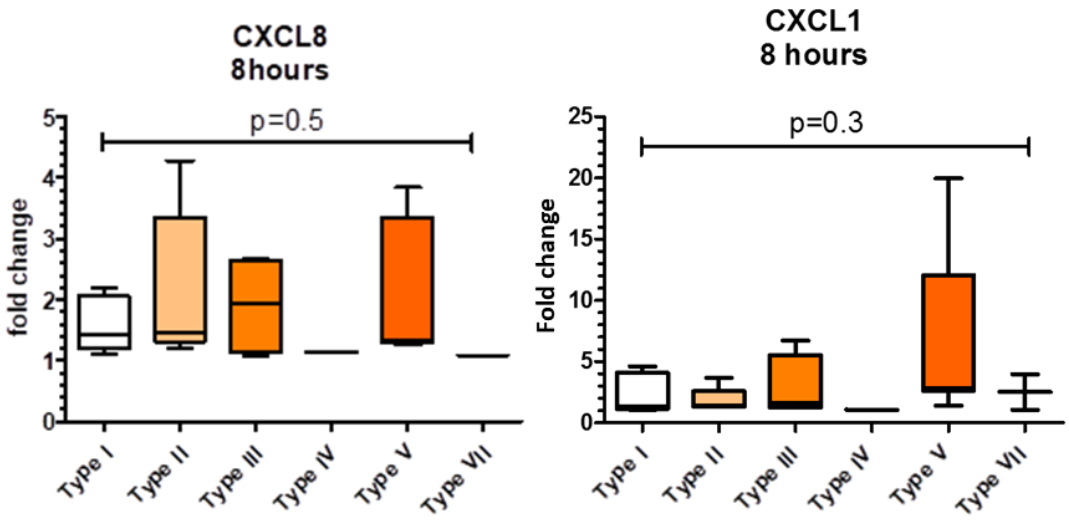


Figure 43. CXCL8 and CXCL1 production from 16HBE cells after co-cultured with 10^7 cfu/ml of different subtypes of *H. parainfluenzae*. 16HBE cells were co-cultured with 10^7 cfu/mL type I *H. parainfluenzae* (n=5), type II *H. parainfluenzae* (n=5), type III *H. parainfluenzae* (n=5), type IV *H. parainfluenzae* (n=1), type V *H. parainfluenzae* (n=5) and type VII *H. parainfluenzae* (n=1) for 8 hours. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IRQ. The line inside the box is median. Y axis shows fold change of CXCL8 level compare to negative control. P-value was calculated by 1 way ANOVA.

3.4.14 Heat-killed *H. parainfluenzae* triggered significant inflammatory responses on 16HBE cells

LPS is the main virulence factor of *Haemophilus* species. To confirm this, we co-cultured 16HBE cells with 6 different clinical strains of heat-killed *H. parainfluenzae*, NTHi and *P. aeruginosa* for 8 hours.

Heat-treated *H. parainfluenzae* and NTHi induced significant CXCL8 production (p=0.053 and p=0.08 respectively). Compared to non-heat-treated *H. parainfluenzae*, NTHi and *P. aeruginosa*; the heat-treated bacteria shows a stronger ability at inducing CXCL8 production in 16HBE cells, although it is not statistically significant (p=0.053, p=0.08 and p=0.37 respectively) (Figure 44).

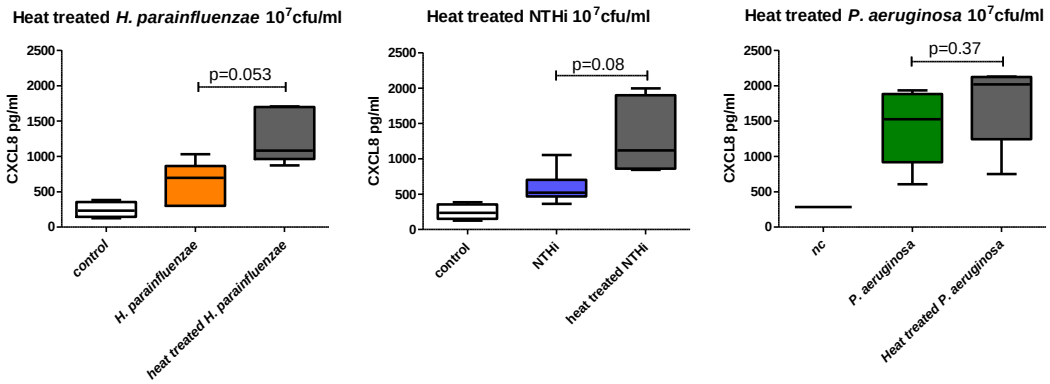
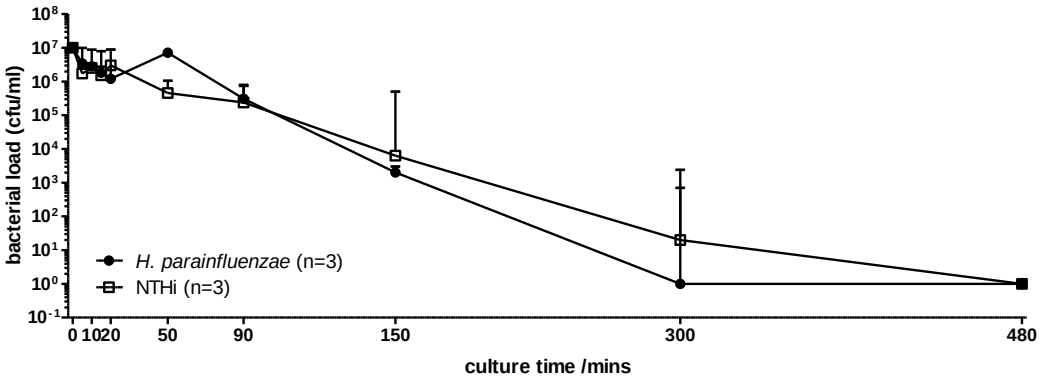


Figure 44. CXCL8 production from 16HBE cells after co-cultured with heat treated *H. parainfluenzae*, NTHi and *P. aeruginosa*. 16HBE cells were co-cultured with 10^7 cfu/mL non heat-treated *H. parainfluenzae* (n=6), heat-treated *H. parainfluenzae* (n=6), non-heat-treated NTHi (n=6), heat-treated NTHi (n=6), non-heat-treated *P. aeruginosa* and heat-treated *P. aeruginosa* for 8 hours. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual CXCL8 level. P-value was calculated by paired t-test.

3.4.15 *H. parainfluenzae* viability after co-cultured with 16HBE cells

Besides the inflammatory responses from 16HBE cells induced by *H. parainfluenzae*. We also looked into the bacterial viability of *H. parainfluenzae* after co-cultured with 16HBE cells. 10^7 cfu/mL *H. parainfluenzae* (n=3) and NTHi(n=3) were co-cultured with 16HBE cells for 10 minutes, 20 minutes, 50 minutes, 90 minutes, 150 minutes and 300 minutes and 480 minutes. There was no significant difference in the outer cellular bacterial load between these two *Haemophilus* species. After 90 minutes co-cultured with 16HBE cells, the number of *H. parainfluenzae* and NTHi both decreased. After 480 minutes (8 hours), both bacterial loads dropped to 0 (Figure 45 a). As background control, 10^7 cfu/mL *H. parainfluenzae* (n=3) or NTHi (n=3) were cultured in cell media without 16HBE cells for 8 hours. After 8 hours, bacterial loads of NTHi and H. *parainfluenzae* did not significantly change compared to the original bacterial load (10^7 cfu/mL) (Figure 45 b).

a) With 16HBE cells



b) Without 16HBE cells

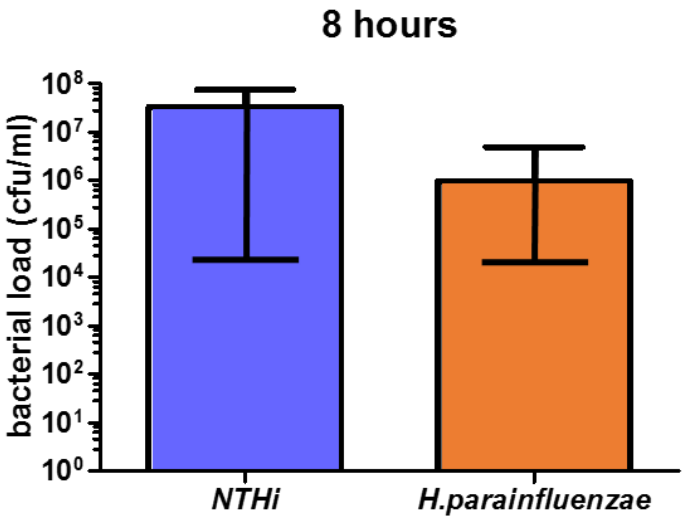


Figure 45. *H. parainfluenzae* viability after co-cultured with 16HBE cells. a) 16HBE cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae* (n=3) or NTHi (n=3)for 10 minutes, 20 minutes, 50 minutes, 90 minutes, 150 minutes and 300 minutes and 480 minutes. b) 10^7 cfu/mL *H. parainfluenzae* (n=3) or NTHi (n=3) were cultured in cell media without 16HBE cells for 8 hours. Cell culture supernatant was collected and diluted, 100 µL diluted supernatant was plated on CBA plates. Bacterial load was calculated according to colony numbers on the plates after 24 hours incubation. Y axis shows bacterial load for *H. parainfluenzae* and NTHi.

3.4.16 NHBE cells' CXCL8 production induced by *H. parainfluenzae* was culture time-dependent

Besides 16HBE cells, we also looked into how primary human bronchial epithelial cells reacted to *H. parainfluenzae*. 6 different clinical strains of *H. parainfluenzae*, NTHi and *P. aeruginosa* were co-cultured with NHBE cells.

NHBE cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae*, NTHi or *P. aeruginosa* for 2 hours, 4 hours, 6 hours and 8 hours. 1-way ANOVA test shows a significant overall increase of CXCL8 with time increasing (*H. parainfluenzae* p=0.04, NTHi p=0.01 and *P. aeruginosa* p=0.016 respectively). CXCL8 production at 8 hours was significantly higher than at 2 hours for *H. parainfluenzae*, NTHi and *P. aeruginosa* groups (p=0.004, p=0.035 and p=0.014 respectively). The median CXCL8 fold change for *H. parainfluenzae* at 2 hours was 0.9, at 4 hours was 0.99, at 6 hours was 1.0 and at 8 hours was 1.2; for NTHi at 2 hours was 0.9, at 4 hours was 0.89, at 6 hours was 1.18 and at 8 hours was 1.12; for *P. aeruginosa* at 2 hours was 0.9, at 4 hours was 1.5, at 6 hours was 1.47 and at 8 hours was 1.9. (Figure 46)

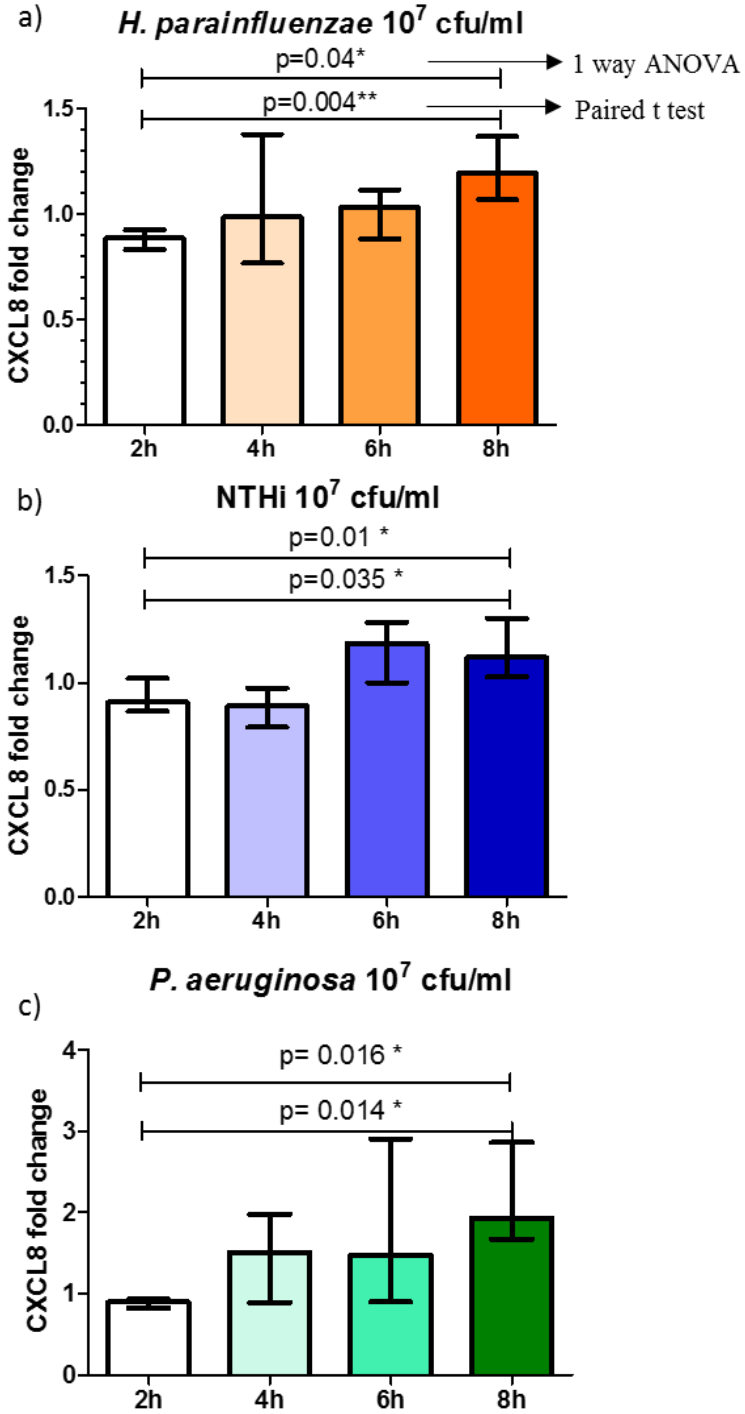


Figure 46. CXCL8 production from NHBE cells after co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* for 2 hours, 4 hours, 6 hours and 8 hours. NHBE cells were co-cultured with a) 10^7 cfu/mL *H. parainfluenzae*, b) 10^7 cfu/mL NTHi and c) 10^7 cfu/mL *P. aeruginosa* (n=6) for 2 hours, 4 hours, 6 hours and 8 hours, control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 compared to the control group. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was 1 way ANOVA.

3.4.17 NHBE CXCL1 production induced by *H. parainfluenzae* was culture time related

We also measured CXCL1 production. Similarly, after NHBE cells co-cultured with *H. parainfluenzae*, NTHi or *P. aeruginosa* for a different time, 1-way ANOVA test shows a significant overall increase of CXCL1 production with time increasing (p=0.0018, p=0.007 and p=0.003 respectively). Different from CXCL8, CXCL1 production was more significant at 6 hours rather than at 8 hours when compared to 2 hours (p=0.006, p=0.0004 and p=0.005 respectively). The median CXCL1 fold change for *H. parainfluenzae* at 2 hours was 1.1, at 4 hours was 0.94, at 6 hours was 1.5 and at 8 hours was 1.3; for NTHi at 2 hours was 1.1, at 4 hours was 0.93, at 6 hours was 1.35 and at 8 hours was 1.28; for *P. aeruginosa* at 2 hours was 1.01, at 4 hours was 1.20, at 6 hours was 1.93 and at 8 hours was 1.40 (Figure 47).

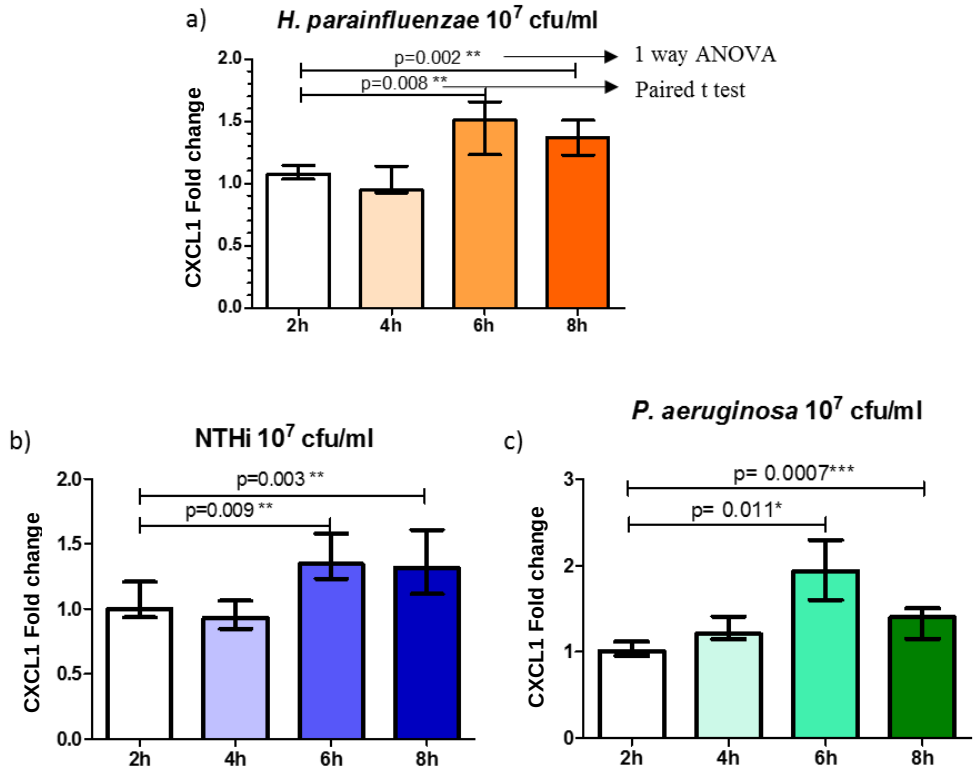


Figure 47. CXCL1 production from NHBE cells after co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* for 2 hours, 4 hours, 6 hours and 8 hours. NHBE cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae*, NTHi and *P. aeruginosa* (n=6) for 2 hours, 4 hours, 6 hours and 8 hours, control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows change fold of CXCL1 compare to control group. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was 1 way ANOVA.

3.4.18 *H. parainfluenzae* induced significant inflammatory responses from NHBE in a similar manner as NTHi and *P. aeruginosa*

H. parainfluenzae (n=6) induced significant more CXCL8, CXCL1 and ICAM-1 when co-cultured with NHBE cells at 10^7 cfu/mL compared to 10^4 cfu/mL (CXCL8 p=0.009, CXCL1 p=0.04 and ICAM p=0.04 respectively). 10^7 cfu/mL *H. parainfluenzae* also induced significantly higher CXCL8, CXCL1 and ICAM-1 production compared to the control group (CXCL8 p=0.02, CXCL1 p=0.033 and ICAM p=0.02 respectively). The median CXCL8 value for 10^7 cfu/mL *H. parainfluenzae* group was 249.1 pg/mL, for 10^4 cfu/mL *H. parainfluenzae* group was 155.4 pg/mL, for the control group was 134.5 pg/mL. The median CXCL1 value for 10^7 cfu/mL group was 1898 pg/mL, for 10^4 cfu/mL group was 1663pg/mL, for the control group was 1527 pg/mL. The median ICAM-1 value for 10^7 cfu/mL group was 1077 pg/mL, for 10^4 cfu/mL group was 681.1 pg/mL, for the control group was 528.4 pg/mL (Figure 48a).

P. aeruginosa (n=6) also induced significantly higher CXCL8, CXCL1 and ICAM-1 when co-cultured with NHBE cells in 10^7 cfu/mL compared to the control group (CXCL8 p=0.017, CXCL1 p=0.027 and ICAM-1 p=0.025 respectively) (Figure 47b).

NTHi (n=6) induced significant higher CXCL8 and ICAM-1 when co-cultured with NHBE cells in 10^7 cfu/mL compared to the control group (CXCL8 p=0.04, ICAM-1 p=0.036). However, we did not observe a similar statistically significant induction for CXCL1, p=0.08 (Figure 47c).

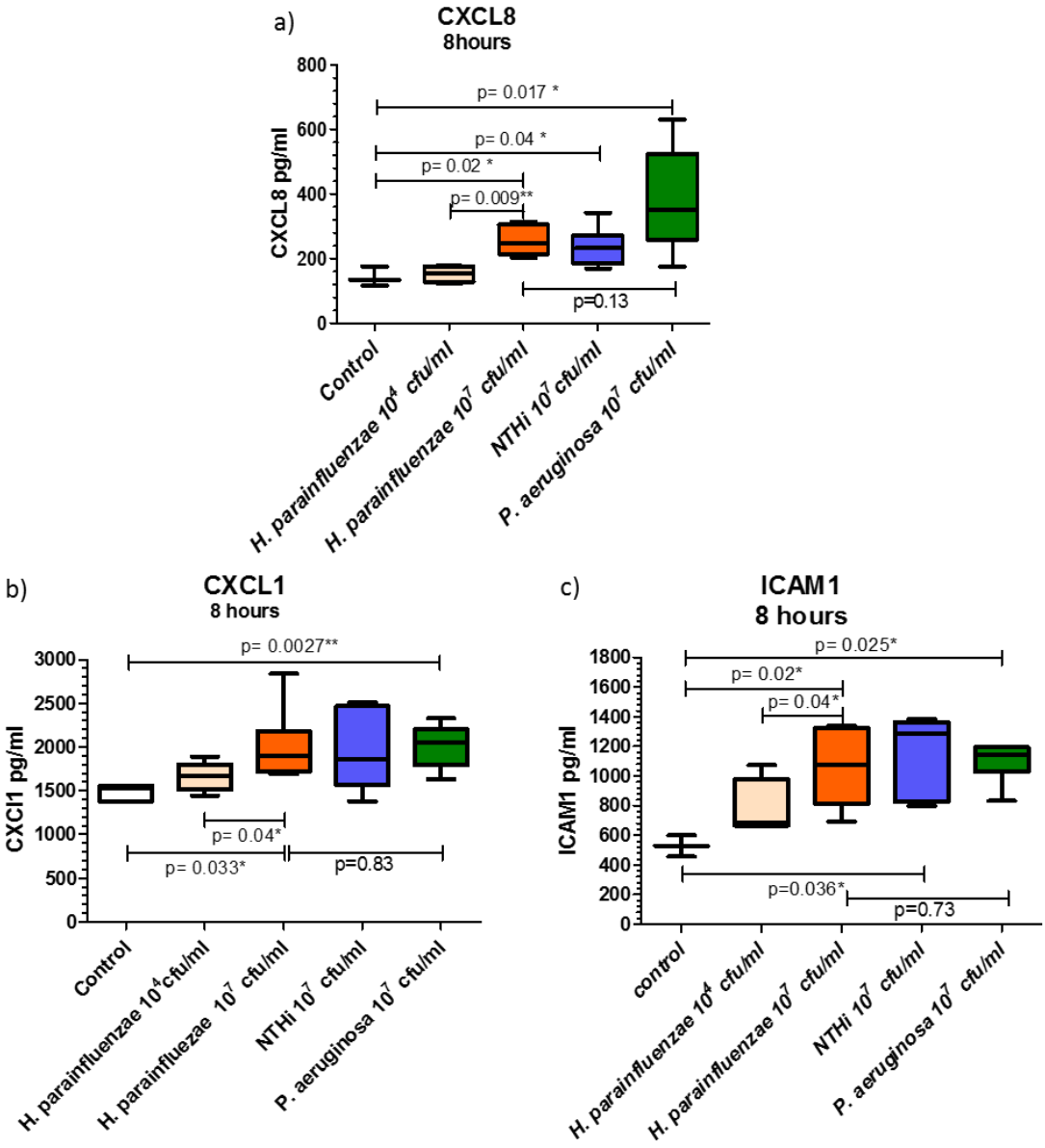


Figure 48. CXCL8, CXCL1 and ICAM-1 production from NHBE cells after co-cultured with 10^7 cfu/mL *H. parainfluenzae*, NTHi and *P. aeruginosa*. NHBE cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=6), 10^7 cfu/mL *H. parainfluenzae* (n=6), 10^7 cfu/mL NTHi (n=6) and 10^7 cfu/mL *P. aeruginosa* (n=6) for 8 hours. Control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. Data is presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of CXCL8, CXCL1 and ICAM-1. P-value was calculated by unpaired t-test between individual groups and 1 way ANOVA between 10^7 cfu/mL *H. parainfluenzae*, 10^7 cfu/mL NTHi and 10^7 cfu/mL *P. aeruginosa* groups.

3.4.19 Patients' primary human nasal epithelial cells

We have observed that *H. parainfluenzae* induced significant cell inflammatory responses from the 16HBE cell line and NHBE cells. We wanted to see if it would have a similar effect on bronchiectasis patient primary cells, so patient primary nasal primary cells were collected from patients in the Royal Infirmary and cultured in the laboratory, and eventually co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa*.

Ten patients were recruited from the bronchiectasis clinic, they were all more than 18 years old, have been clinically stable for at least 21 days, they did not have severe asthma, rhinitis (require nasal steroid), and not on anti-coagulants. They were not current smokers or ex-smokers for less than one year.

3.4.19.1 Patients' primary human nasal epithelial cells culture

After cells were collected and cultured in cell media, cells usually started to attach to the plates and had initial growth within two days. Figure 49 shows the image of cells after cultured for four days and seven days.

There was a difference in the growth speed between different samples. Five strains of cells out of ten patients' samples survived until passage 2. Bacteria culture experiments were done at passage 2. After passage 3, cells started to became swollen and grew at slow speed or even stopped growing.

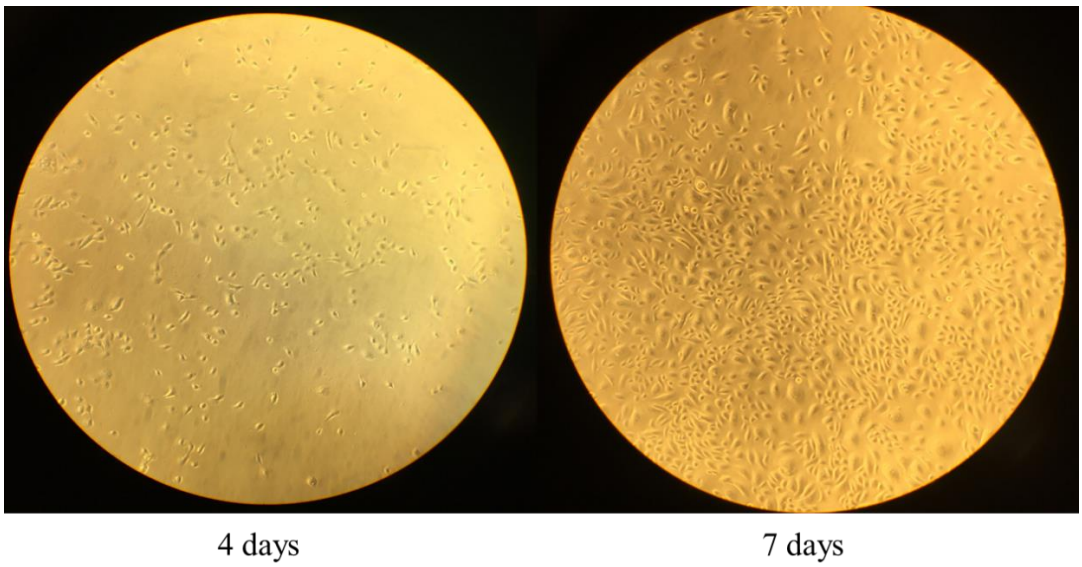


Figure 49. Patients' primary human nasal epithelial cells under microscope at 10×10 magnification. Patients' primary human nasal epithelial cells were cultured for 4 days and 7 days.

3.4.19.2 Patients' primary human nasal epithelial cells' CXCL8 and CXCL1 production induced by *H. parainfluenzae* was culture time-dependent

Five patients' nasal epithelial cell strains were co-cultured with 3 different clinical *H. parainfluenzae* strains at 10^7 cfu/mL for 2 hours, 4 hours, 6 hours and 8 hours. 1-way ANOVA test shows a significant overall increase of CXCL8 and CXCL1 levels with time (p=0.019 and p=0.02 respectively). CXCL8 and CXCL1 production at 8 hours was significantly higher than at 2 hours (p=0.014 and p=0.014 respectively). The median CXCL8 fold change at 2 hours was 1.02, at 4 hours 1.01, at 6 hours 1.2 and at 8 hours 1.35; fold changes for CXCL1 were: at 2 hours 1.02, at 4 hours 1.01, at 6 hours 1.2 and at 8 hours 1.6 (Figure 50).

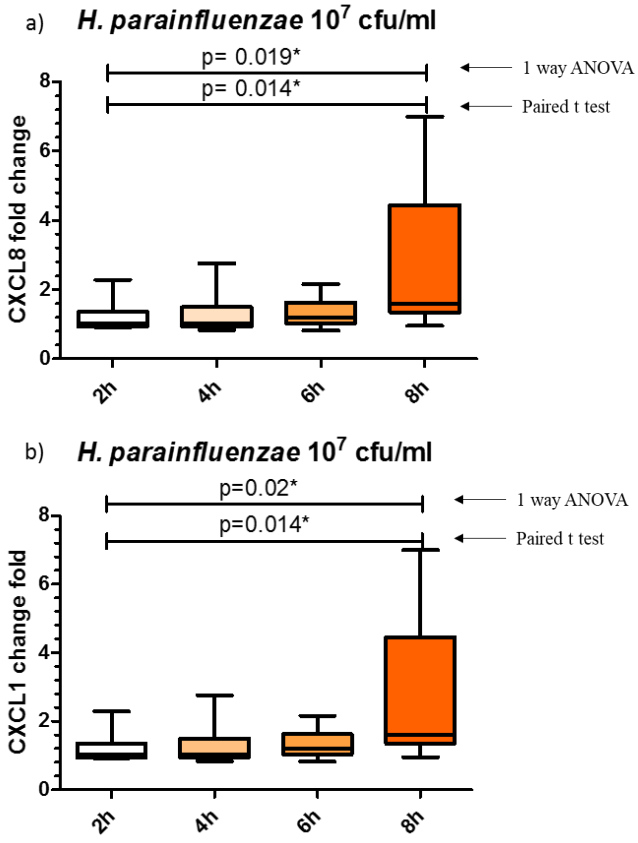


Figure 50. CXCL8 and CXCL1 production from patients' nasal epithelial cells after co-cultured with *H. parainfluenzae* for 2 hours, 4 hours, 6 hours and 8 hours. Five strains of patients' primary human nasal epithelial cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae* (n=3) for 2 hours, 4 hours, 6 hours and 8 hours, control was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. a) and b) show the CXCL8 and CXCL1 values pooled results from all cells. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 and CXCL1compared to the control group. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was 1 way ANOVA.

3.4.19.3 Patients' primary human nasal epithelial cells CXCL8 and CXCL1 production induced by NTHi was culture time-dependent

Five strains of patients' nasal epithelial cells were co-cultured with 10^7 cfu/mL NTHi (n=3) for 2 hours, 4 hours, 6 hours and 8 hours. 1-way ANOVA test shows a significant overall increase of CXCL8 and CXCL1 levels with time increasing (p=0.04 and p=0.03 respectively). CXCL8 and CXCL1 productions at 8 hours were significantly higher than at 2 hours (p=0.02 and p=0.0006 respectively). At 6 hours, NTHi also can stimulate significant CXCL1 production compared to 2 hours (p=0.03). The median CXCL8 fold change at 2 hours was 1.01, at 4 hours was 1.05, at 6 hours was 1.22 and at 8 hours was 1.57; for CXCL1 at 2 hours was 0.86, at 4 hours was 1.09, at 6 hours was 1.22 and at 8 hours was 1.71 (Figure 51).

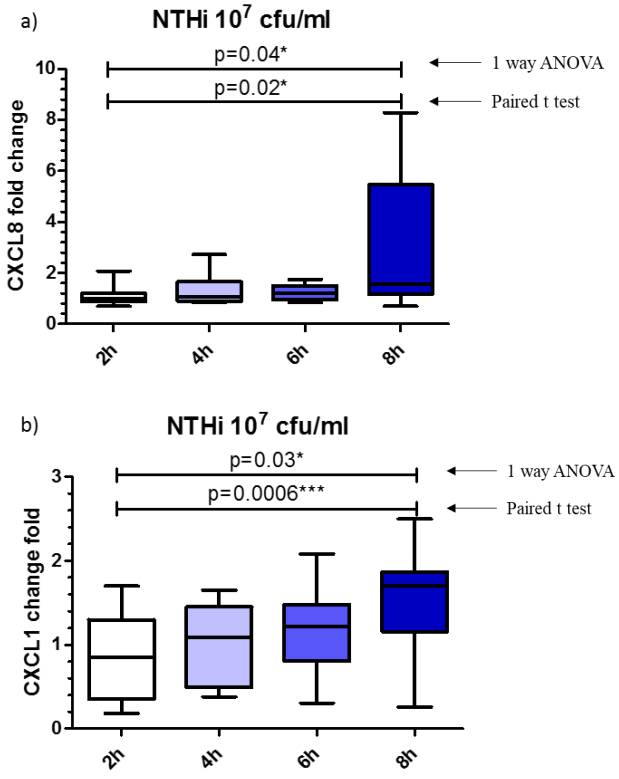


Figure 51. CXCL8 and CXCL1 production from patients' nasal epithelial cells after co-cultured with NTHi for 2 hours, 4 hours, 6 hours and 8 hours. Five strains of patients' primary human nasal epithelial cells were co-cultured with 10^7 cfu/mL NTHi (n=3) for 2 hours, 4 hours, 6 hours and 8 hours, control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. a) and b) show the CXCL8 and CXCL1 values from pooled results from all cells. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 and CXCL1 compared to control group. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was calculated using 1 way ANOVA.

3.4.19.4 Patients' primary human nasal epithelial cells CXCL8 and CXCL1 production induced by *P. aeruginosa* was culture time-dependent

Five strains of patients' nasal epithelial cells were co-cultured with 10^7 cfu/mL *P. aeruginosa* (n=3) for 2 hours, 4 hours, 6 hours and 8 hours. 1-way ANOVA test shows a significant overall increase of CXCL8 and CXCL1 level with time increasing (p=0.02 and p=0.004 respectively). CXCL8 and CXCL1 productions at 8 hours were significantly higher than at 2 hours (p=0.01 and p=0.0009 respectively). *P. aeruginosa* also can trigger significantly more CXCL8 production at 4 hours and 6 hours compared to 2 hours (p=0.02, p=0.003). The median CXCL8 fold change for at 2 hours was 1.26, at 4 hours was 1.75, at 6 hours was 3.20 and at 8 hours was 3.32; for CXCL1 at 2 hours was 1.25, at 4 hours was 1.30, at 6 hours was 1.86 and at 8 hours was 2.10 (Figure 52).

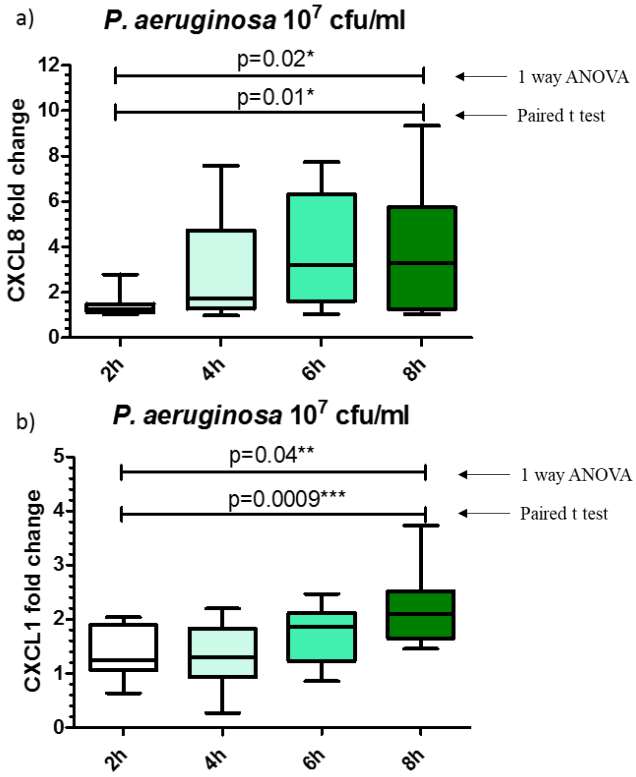


Figure 52. CXCL8 and CXCL1 production from patients' nasal epithelial cells after co-cultured with *P. aeruginosa* for 2 hours, 4 hours, 6 hours and 8 hours. Five strains of patients' primary human nasal epithelial cells were co-cultured with 10^7 cfu/mL *P. aeruginosa* (n=3) for 2 hours, 4 hours, 6 hours and 8 hours, control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. a) and b) show the CXCL8 values from individual cells. c) shows the pooled results from all cells. Data is presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows fold change of CXCL8 compare to control group. P-values between individual time points were calculated by paired t-test. P-value for overall comparison was calculated using 1 way ANOVA.

3.4.19.5 *H. parainfluenzae* induced inflammatory responses in patients' nasal epithelial cells in a similar manner as NTHi and *P. aeruginosa*

H. parainfluenzae (n=3) at 10^7 cfu/mL induced significantly more CXCL8, CXCL1 and ICAM-1 production when co-cultured with five different strains of patients' nasal epithelial cells compare to the negative control. Similarly, to other epithelial cells (CXCL8 p=0.038, CXCL1 p=0.0005, ICAM-1 p=0.0001). There was significantly more CXCL8, CXCL1 and ICAM-1 production when cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae* compared 10^4 cfu/mL of *H. parainfluenzae* (CXCL8 p=0.04, CXCL1 p=0.03 and ICAM-1 p=0.0001 respectively). 10^4 cfu/mL *H. parainfluenzae* did not induce significant CXCL8, CXCL1 and ICAM-1 production compared to the control group. The median CXCL8 value for 10^7 cfu/mL *H. parainfluenzae* group was 319.9 pg/mL, for 10^4 cfu/mL *H. parainfluenzae* group was 171.6 pg/mL, for control group was 187.1 pg/mL. The median CXCL1 value for 10^7 cfu/mL group was 1271 pg/mL, for 10^4 cfu/mL group was 1048 pg/mL, for control group was 777.3 pg/mL. The median ICAM-1 value for 10^7 cfu/mL group was 315.4 pg/mL, for 10^4 cfu/mL group was 249 pg/mL, for control group was 183.1 pg/mL (Figure 53).

P. aeruginosa (n=3) also induced significantly more CXCL8, CXCL1 and ICAM-1 production when co-culture with patients' primary human nasal epithelial cells (n=5) at 10^7 cfu/mL compared the negative control (CXCL8 p=0.017, CXCL1 p=0.0002 and ICAM-1 p=0.0001 respectively). P-value was calculated using an unpaired t-test (Figure 53).

NTHi (n=3) induced significantly more CXCL1 and ICAM-1 production from patients' primary human nasal epithelial cells (n=5) at 10^7 cfu/mL (CXCL1 p=0.004 and ICAM-1 p=0.0002 respectively). P-value was calculated by unpaired t-test (Figure 53).

There was no significant difference in the CXCL8, CXCL1 and ICAM-1 level when patients' primary human nasal epithelial cells were co-cultured with 10^7 cfu/mL *H. parainfluenzae,* NTHi or *P. aeruginosa*.

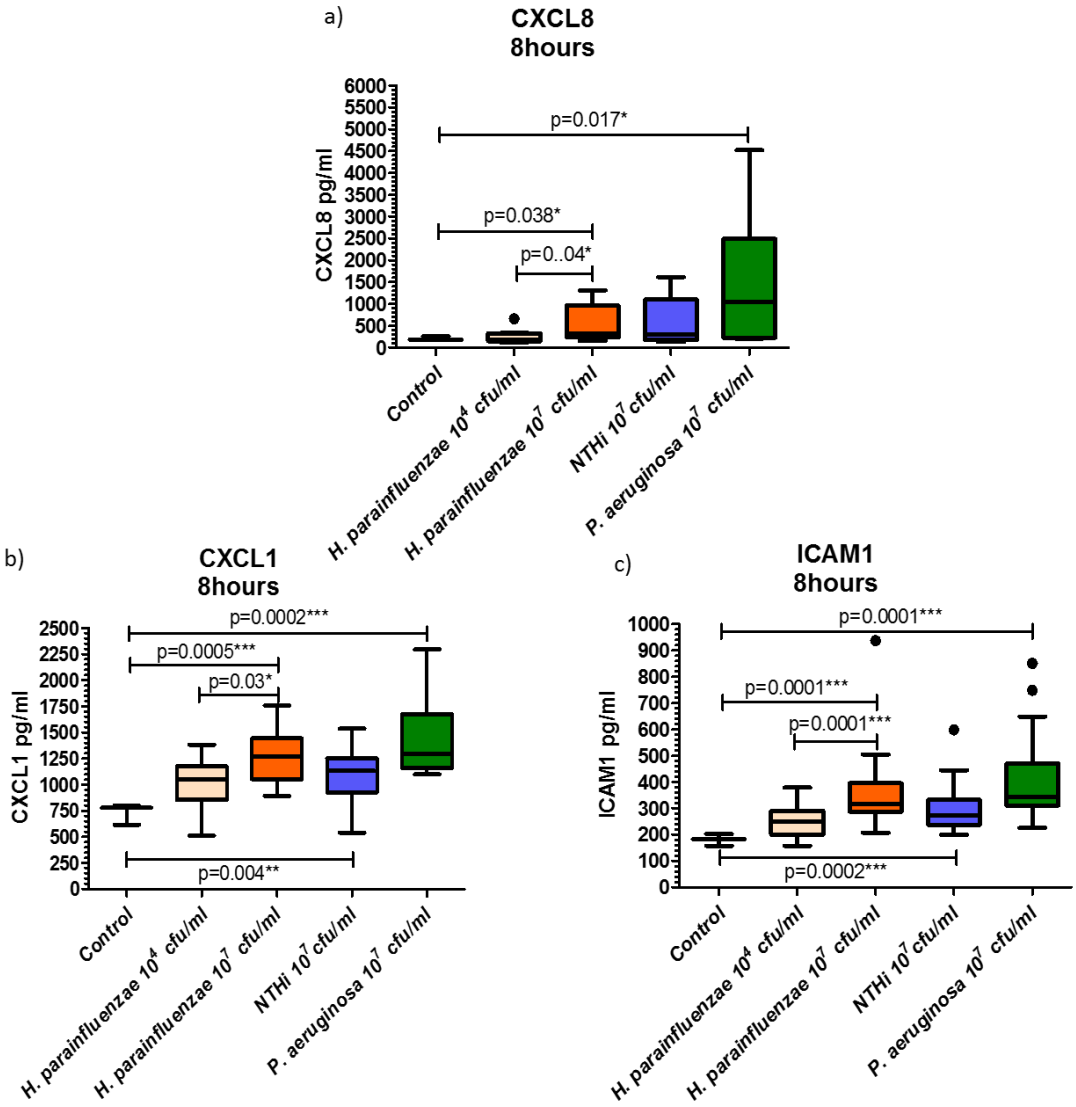


Figure 53. CXCL8, CXCL1 and ICAM-1 production from patients' nasal epithelial cells after co-cultured with *H. parainfluenzae*, NTHi and *P. aeruginosa* for 8 hours. Five strains of patients' nasal epithelial cells were co-cultured with 10^4 cfu/mL *H. parainfluenzae* (n=3), 10^7 cfu/mL *H. parainfluenzae* (n=3), 10^7 cfu/mL NTHi (n=3) and 10^7 cfu/mL *P. aeruginosa* (n=3) for 8 hours. Control group was cultured with same volume of PBS. 100 µL of cell culture supernatant was run on each ELISA. a), b) and c) show the pooled results from all cells. Data are presented in Tukey box and whisker plots. The box shows +/- IQR. The line inside the box is median. Y axis shows actual value of CXCL8 and CXCL1. P-value was calculated using unpaired t-test between individual groups and 1 way ANOVA between 10^7 cfu/mL *H. parainfluenzae*, 10^7 cfu/mL NTHi and 10^7 cfu/mL *P. aeruginosa* groups.

3.4.20 Conclusion for aim 4

- According to the microarray results, *H. parainfluenzae* at 10^7cfu/mL stimulated wide range of inflammatory responses in 16 HBE cell after 8 hours of culture.
- There was a dose-response from 10^4 cfu/mL to 10^8 cfu/mL for *H. parainfluenzae,* with significant increased CXCL8, CXCL1, LCN2, ICAM-1 level at 10^7cfu/mL, no difference in IL1β production consistent to the microarray results
- The inflammatory response was time-dependent. At 8 hours, *H. parainfluenzae* was similar to NTHi and *P. aeruginosa* at stimulating CXCL8 production, with a low cell death rate.
- Heat-treated *H. parainfluenzae* stimulated higher CXCL8 production than non-heat-treated *H. parainfluenzae*.
- *H. parainfluenzae* subtypes stimulated similar inflammatory responses, although numbers were low in type IV and VII.
- Similar dose-responses from 10^4 cfu/mL to 10^8 cfu/mL of *H. parainfluenzae* on NHBE cells and primary human nasal epithelial cells were observed.
- Primary human nasal epithelial cells were challenging to grow, we had a 50% success rate.
- Overall, we showed *H. parainfluenzae* in high bacterial loads can stimulate significant inflammatory responses from epithelial cells.

3.5 Results for aim 5. Assessing the impact of coinfection of *H. parainfluenzae* with *P. aeruginosa* and *H. parainfluenzae* with NTHi

This part of the study was attempted to discover how microbiome or bacterial interaction impact epithelial cells inflammatory response.

To understand multi pathogen colonisation in bronchiectasis, we analysed sputum microbiology results from 208 bronchiectasis patients. Those patients were all ≥ 18 years old and had stable bronchiectasis.

We then looked into how *H. parainfluenzae* and *P. aeruginosa* together would change 16HBE cell inflammatory responses compared to single bacteria interaction with epithelial cells.

3.5.1 In bronchiectasis, 20% of patients have more than one pathogen colonisation

In 208 bronchiectasis patients, 169 of them had single dominant pathogen isolated from sputum samples. There were 39 patients had more than one predominant pathogen (34 double pathogens, five triple pathogens). In those 39 patients, *Haemophilus* species and *S. pneumoniae* was the most prevalent combination (n=16), followed by *Haemophilus* species with *Moraxella catarrhalis* (n=11). *P. aeruginosa* was found to co-exist with *S. pneumonia* (n=4) and *Moraxella catarrhalis* (n=1). Interestingly, as the two most common pathogen, *Haemophilus* species had not been found co-exist with *P. aeruginosa*. *P. aeruginosa*, is well known for being able to interact with other bacteria in multiple strategies, so we decided to look into bacterial interaction between *H. parainfluenzae* with *P. aeruginosa*.

The other co-exist dominant pathogens were: *Serratia marcens* with *Escherichia coli*, *Acinetobacter* with *Stenotrophomonas maltophilia* (*S. maltophilia*), yeast with *S. pneumoniae*, *S. maltophilia* with *Klebsiella oxytoca*, *P. aeruginosa* with *Eschericia coli* and Mould with *Serratia marcescens*. (Figure 54)

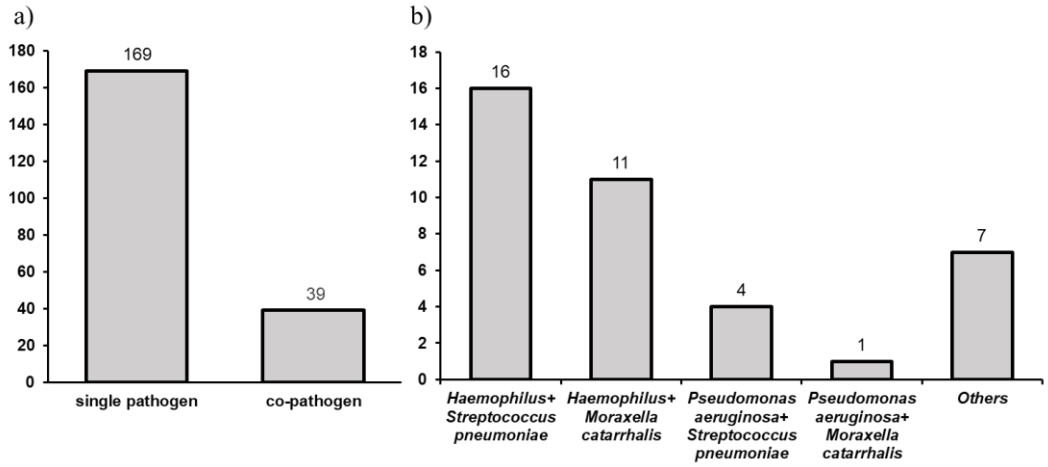


Figure 54. Co-pathogen in bronchiectasis patients. a) 169 (81.2%) patients had single pathogen from their sputum samples. 39 (18.8%) patients had more than one pathogen. b) among these 39 patients, 16 (41%) patients had *Haemophilus* species with S. *pneumoniae*, 11(28%) patients had *Haemophilus* species; 4 (2.3%) patients had *P. aeruginosa* and *S. pneumonia*. 1 (2.5%) patients had *P. aeruginosa* and *M. catarrhalis.* There was 7 (4.1%) patients had other pathogens.

3.5.2 *H. parainfluenzae* and *P. aeruginosa* co-cultured with 16HBE cells for 8 hours

16HBE cells were cultured with either *H. parainfluenzae* (n=12) at concentrations of 1×10^5 cfu/mL and 2×10^5 cfu/mL or *P. aeruginosa* (n=12) at 1×10^5 cfu/mL and 2×10^5 cfu/mL, as well as with both *H. parainfluenzae* (n=12) at 1×10^5 cfu/mL and *P. aeruginosa* (n=12) at 1×10^5 cfu/mL for 8 hours. CXCL8, CXCL1 and LCN2 production was measured after 8 hours. In the case of CXCL8 production, there was a significant decrease when *P. aeruginosa* was co-cultured with *H. parainfluenzae* in 16HBE cells compared to when *P. aeruginosa* was cultured on its own at 1×10^5 cfu/mL or 2×10^5 cfu/mL (p=0.0007 and p=0.0005 respectively). The median CXCL8 value of the 1×10^5 cfu/mL *H. parainfluenzae* group was 1206 pg/mL, the 2×10^5 cfu/mL *H. parainfluenzae* group 1110 pg/mL, 1×10^5 cfu/mL *P. aeruginosa* group 1450 pg/mL, 2×10^5 cfu/mL *P. aeruginosa* group 1399 pg/mL, and the mixed *H. parainfluenzae* and *P. aeruginosa* at 1×10^5 cfu/mL group 1150 pg/mL (Figure 55).

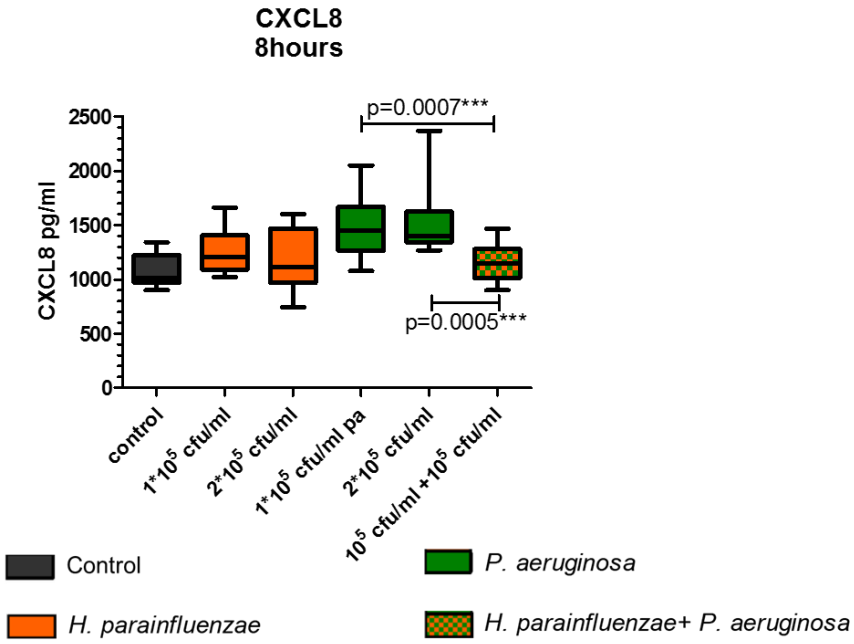


Figure 55. CXCL8 production from 16HBE cells after co-cultured with *H. parainfluenzae* and *P. aeruginosa* together for 8 hours. 16HBE cells were cultured with 1×10^5 cfu/mL and 2×10^5 cfu/mL *H. parainfluenzae* (n=12), 1×10^5 cfu/mL and 2×10^5 cfu/mL *P. aeruginosa* (n=12), and 1×10^5 cfu/mL *H. parainfluenzae* (n=12) together with 1×10^5 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was cultured with same volume of PBS. After 8 hours, media was then collected. Media CXCL8 level was measured by ELISA. Data are presented in box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8.

Similar results were found in CXCL1 and LCN2 level. There was a significant decrease when 1×10^5 cfu/mL *P. aeruginosa* co-culture with 16HBE cells together with 1×10^5 cfu/mL *H. parainfluenzae* compared to 10^5 cfu/mL *P. aeruginosa* and 2×10^5 cfu/mL *P. aeruginosa* co-cultured with 16HBE cells on its own (CXCL1 p=0.003, p=0.0003, LCN2 p=0.04 and p=0.003 respectively). The median CXCL1 value 1×10^5 cfu/mL *H. parainfluenzae* group was 242.6 pg/mL, 2×10^5 cfu/mL *H. parainfluenzae* group was 235.3 pg/mL, 1×10^5 cfu/mL *P. aeruginosa* group was 313.9 pg/mL, 2×10^5 cfu/mL *P. aeruginosa* group was 334.7 pg/mL, for 1×10^5 cfu/mL *H. parainfluenzae* together with 1×10^5 cfu/mL *P. aeruginosa* group was 263.9 pg/mL (Figure 56a). The median LCN2 value 1×10^5 cfu/mL *H. parainfluenzae* group was 53.8 pg/mL, 2×10^5 cfu/mL *H. parainfluenzae* group was 33.9 pg/mL, 10^5 cfu/mL *P. aeruginosa* group was 87.1 pg/mL, 2×10^5 cfu/mL *P. aeruginosa* group was 159.4 pg/mL, for 10^5 cfu/mL *H. parainfluenzae* together with 1×10^5 cfu/mL *P. aeruginosa* group was 40.88 pg/mL (Figure 56b).

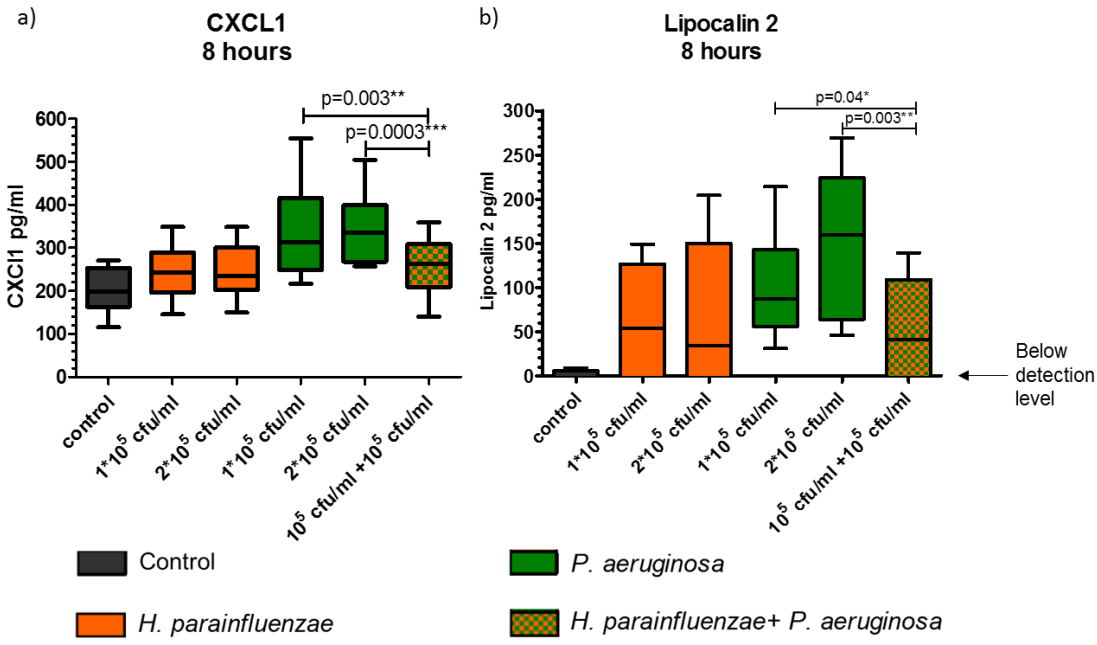


Figure 56. **CXCL1 and LCN2 production from 16HBE cells after co-cultured with *H. parainfluenzae* and *P. aeruginosa* together for 8 hours.** 16HBE cells were cultured with 10^5 cfu/mL and 2×10^5 cfu/mL *H. parainfluenzae* (n=12), 1×10^5 cfu/mL and 2×10^5 cfu/mL *P. aeruginosa* (n=12), and 1×10^5 cfu/mL *H. parainfluenzae* (n=12) together with 1×10^5 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was cultured with same volume of PBS. After 8 hours, media was then collected. Media CXCL1and LCN2 levels were measured by ELISA. Data are presented in box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL1 and LCN2.

3.5.3 *H. parainfluenzae* and *P. aeruginosa* co-cultured with 16HBE cells together for 8 hours in a higher bacterial load

16HBE cells were cultured with 1×10^6 cfu/mL and 2×10^6 cfu/mL *H. parainfluenzae* (n=12), 1×10^6 cfu/mL and 2×10^6 *P. aeruginosa* (n=12), and 1×10^6 cfu/mL *H. parainfluenzae* (n=12) together with 1×10^6 cfu/mL *P. aeruginosa* (n=12) for 8 hours.

In the combination group, there was no significant decrease of CXCL8 production compared to 1×10^6 cfu/mL and 2×10^6 cfu/mL *P. aeruginosa* group (p=0.58 and p=0.06 respectively). The median CXCL8 value 1×10^6 cfu/mL *H. parainfluenzae* group was 1567 pg/mL, 2×10^6 cfu/mL *H. parainfluenzae* group was 1701 pg/mL, 1×10^6 cfu/mL *P. aeruginosa* group was 2051 pg/mL, 2×10^6 cfu/mL *P. aeruginosa* group was 2428 pg/mL, for 1×10^6 cfu/mL *H. parainfluenzae* together with 1×10^6 cfu/mL *P. aeruginosa* group was 2230 pg/mL. (Figure 57)

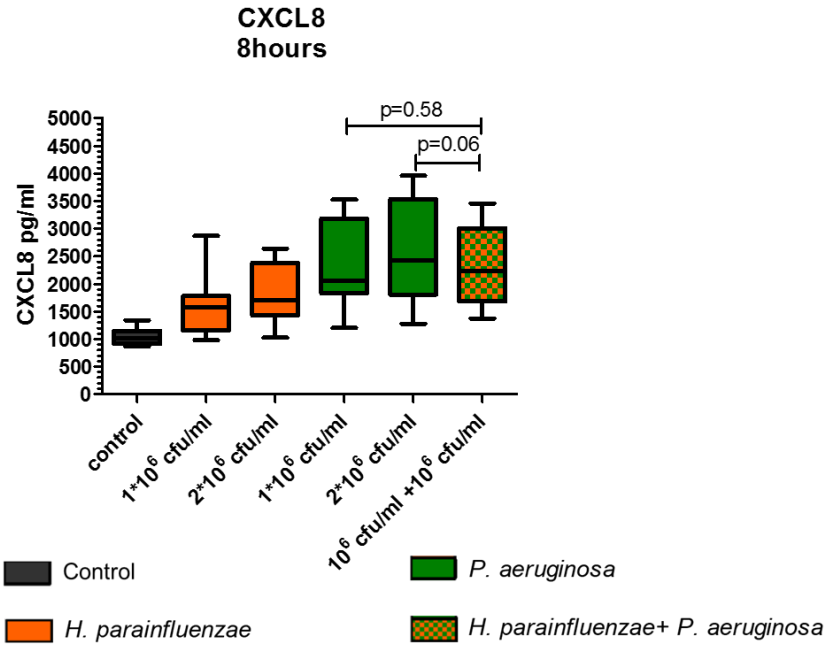


Figure 57. CXCL8 production from 16HBE cells after co-cultured with *H. parainfluenzae* and *P. aeruginosa* together for 8 hours. 16HBE cells were cultured with 10^6 cfu/mL and 2×10^6 cfu/mL *H. parainfluenzae* (n=12), 1×10^6 cfu/mL and 2×10^6 cfu/mL *P. aeruginosa* (n=12), and 1×10^6 cfu/mL *H. parainfluenzae* (n=12) together with 1×10^6 cfu/mL *P. aeruginosa* (n=12) for 8 hours. Control group was same volume of PBS. After 8 hours, media was then collected. Media CXCL8 level was measured by ELISA. Data is presented in box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8.

3.5.4 *H. parainfluenzae* and NTHi co-cultured with 16HBE cells together for 8 hours at different bacterial loads

16HBE cells were cultured with NTHi at different bacterial load (from 10^3 cfu/mL to 10^8 cfu/mL). Those cells were also cultured with 1×10^5 cfu/mL *H. parainfluenzae* or the same volume of PBS. After 8 hours, CXCL8 production was measured, there was no difference between the cells cultured with only NTHi or with both NTHi and *H. parainfluenzae*. (Figure 58)

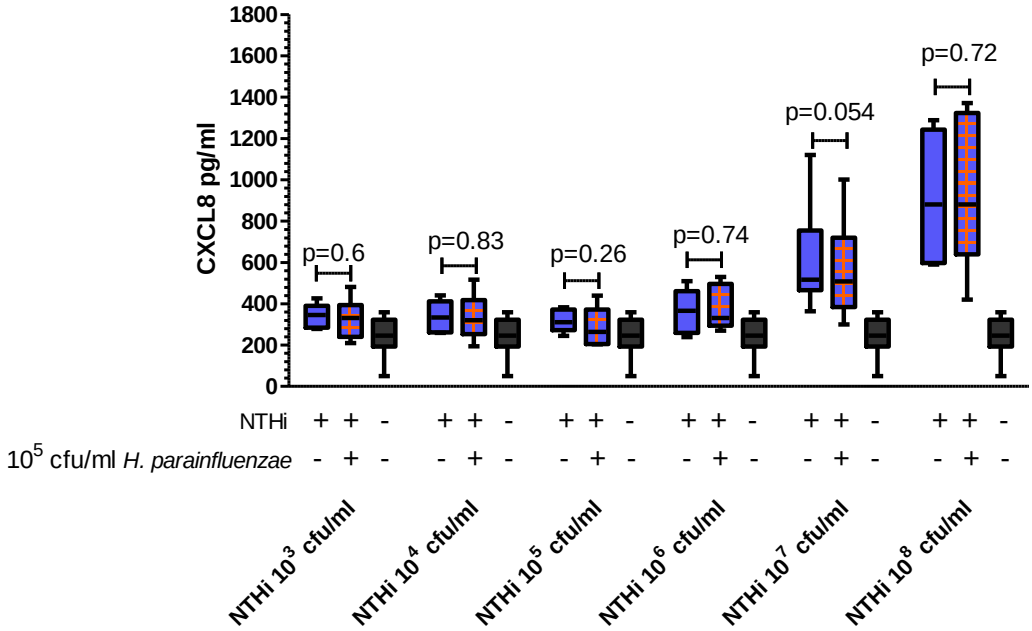


Figure 58. CXCL8 production from 16HBE cells after co-cultured with *H. parainfluenzae* and NTHi together for 8 hours.16HBE cells were cultured with NTHi in different bacterial load (from 10^3 cfu/mL to 10^8 cfu/mL). Those cells were also cultured with 1×10^5 cfu/mL *H. parainfluenzae* (+) or same volume of PBS (-). After 8 hours, media was then collected. Media CXCL8 level was measured by ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8.

3.5.5 Heat-treated *H. parainfluenzae* and *P. aeruginosa* co-cultured with 16HBE cells together

To investigate the mechanism behind the decreased inflammatory responses in the *H. parainfluenzae* and *P. aeruginosa* combined group, we co-cultured 16HBE cells with heat-treated *H. parainfluenzae* (n=6) and *P. aeruginosa* (n=6). 80 ºC water bath for one-hour kills bacteria. However, their LPS are heat-stable endotoxins. The results showed that there was no significant difference in CXCL8 and CXCL1 production between heat-treated *H. parainfluenzae* with heat-treated *P. aeruginosa* combined group and heat-treated *P. aeruginosa* group. This result suggested that the decreasing effect requires living bacteria, which indicate it could be related to bacterial interaction. (Figure 59)

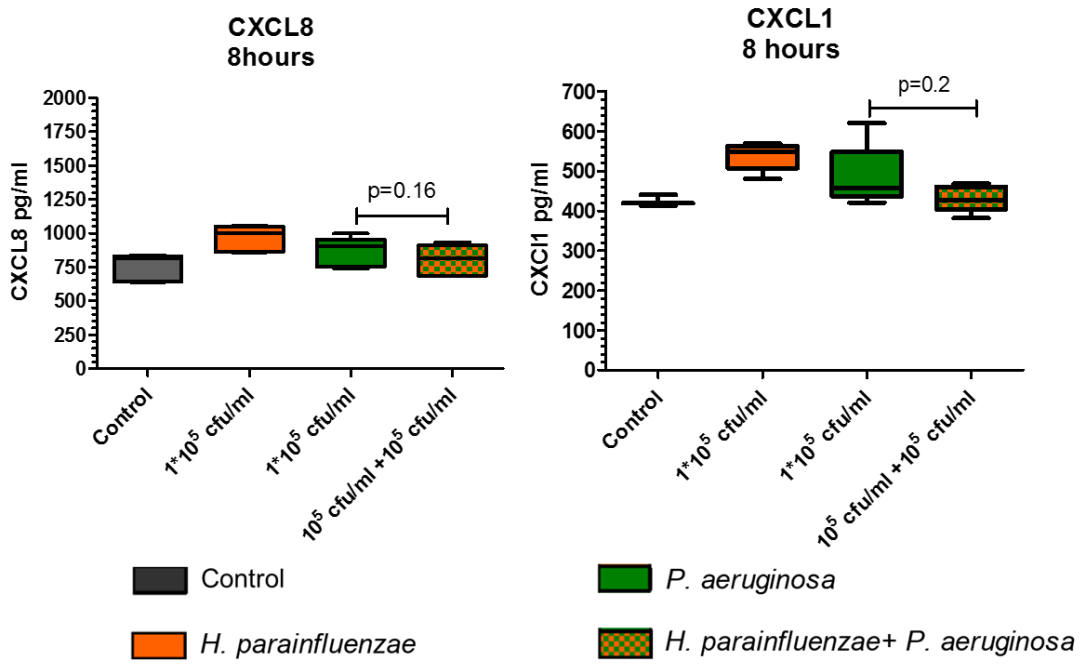


Figure 59. CXCL8 and CXCL1 production from 16HBE cells after co-cultured with heated treated *H. parainfluenzae* and *P. aeruginosa* together for 8 hours.16HBE cells were cultured with heat treated 1×10^5 cfu/mL *H. parainfluenzae* (n=6), 1×10^5 cfu/mL *P. aeruginosa* (n=6), and 10^5 cfu/mL *H. parainfluenzae* (n=6) together with 10^5 cfu/mL *P. aeruginosa* (n=6) for 8 hours. Control group was cultured with same volume of PBS. After 8 hours, media was then collected. Media CXCL8 level was measured by ELISA. Data are presented in box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8 of CXCL1.

3.5.6 *H. parainfluenzae* and non-PCN *P. aeruginosa* co-cultured with 16HBE cells together for 8 hours

PCN is an important virulence factor of *P. aeruginosa*; it interferes with multiple cellular functions in *P. aeruginosa*. We proposed that PCN may play a role in the decreasing effect found in the combined group. 16HBE cells were co-cultured with 1×10^5 cfu/mL 6 different strains of *H. parainfluenzae*, 1×10^5 cfu/mL 6 different strains of non-PCN *P. aeruginosa*, as well as 1×10^5 cfu/mL *H. parainfluenzae* together with 1×10^5 cfu/mL non-PCN *P. aeruginosa* for 8 hours. CXCL8 and CXCL1 were measured.

In the combination group, there was still a significant decrease of CXCL8 (p=0.02) and CXCL1 (p=0.02) production compares to non-PCN *P. aeruginosa* alone group. The median CXCL8 and CXCL1 value for non-PCN *P. aeruginosa* group were 1636 pg/mL and 567 pg/mL, for H. parainfluenzae and non-PCN *P. aeruginosa* group was 1372 pg/mL and 322 pg/mL. This suggests that PCN was not a critical factor (Figure 60).

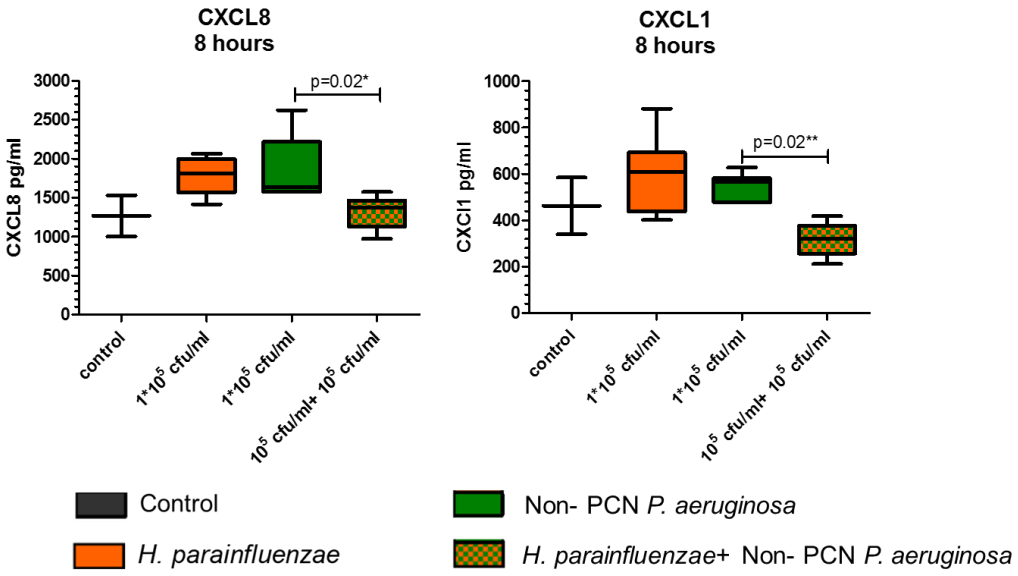


Figure 60. CXCL8 and CXCL1 production from 16HBE cells after co-cultured with *H. parainfluenzae* and non-PCN *P. aeruginosa* together for 8 hours. 16HBE cells were cultured with 1×10^5 cfu/mL *H. parainfluenzae* (n=6), 1×10^5 cfu/mL non PCN *P. aeruginosa* (n=6), and 10^5 cfu/mL *H. parainfluenzae* (n=6) together with 10^5 cfu/mL non PCN *P. aeruginosa* (n=6) for 8 hours. Control group was cultured with same volume of PBS. After 8 hours, media was then collected. Media CXCL1and CXCL8 levels were measured by ELISA. Data are presented in min to max box and whisker plots. The box shows +/- IRQ. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8 and CXCL1.

3.5.7 *H. parainfluenzae* co-cultured with 16HBE cells with media that had cultured cells and *P. aeruginosa*

From previous results, we proposed that bacterial interaction between *P. aeruginosa* and *H. parainfluenzae* may lead to a decrease of CXCL8 and CXCL1 production. Bacterial interaction can be direct interaction or indirect interaction. *P. aeruginosa* are already known for being able to produce outer membrane proteins, outer membrane vesicles and toxins, *P. aeruginosa* are also very well researched about its quorum sensing pathway (Soukarieh F *et al*). Hence, we decided to look into the indirect interaction between *H. parainfluenzae* and *P. aeruginosa*.

With this purpose, we tried to mimic an indirect interaction situation between *H. parainfluenzae* and *P. aeruginosa*. We co-cultured 1×10^5 cfu/mL *H. parainfluenzae* and 16HBE cells in the media that has cultured 16HBE cells and 1×10^5 cfu/mL *P. aeruginosa* (*P. aeruginosa* media). In this way, *H. parainfluenzae* and cells were exposed to proteins or chemicals or DNA that produced by *P. aeruginosa* instead of directly in contact with *P. aeruginosa*. After 8 hours co-culture, the supernatant was collected, and CXCL8 and CXCL1 levels were measured. Results for this group is column C in figure 61.

H. parainfluenzae and 16HBE cells were also cultured in media that has cultured only 16HBE cells (cell media) as a comparison. Results for this group is shown as column A in figure 61.

Control group was 16HBE cells cultured with 1×10^5 cfu/mL *H. parainfluenzae* in fresh media; this group is column E in figure 61. For background control, we cultured cells with those different media without the presence of *H. parainfluenzae*, those groups are column D (with *P. aeruginosa* media), column B (with cell media) and column F (with fresh media) in figure 61.

From CXCL8 and CXCL1 results, 16HBE cells and *H. parainfluenzae* that were cultured in *P. aeruginosa* media (column C) significantly reduced the CXCL8, and CXCL1 production compared to the 16HBE cells and *H. parainfluenzae* that were cultured in cell media group (column A) (CXCL8 $p=0.0017$ and CXCL1 $p=0.027$ respectively). The median CXCL8 value for column C was 2347 pg/mL, for column

A was 2739 pg/mL. The median CXCL1 value for column C was 910.5 pg/mL, for column A was 1210 pg/mL (Figure 61).

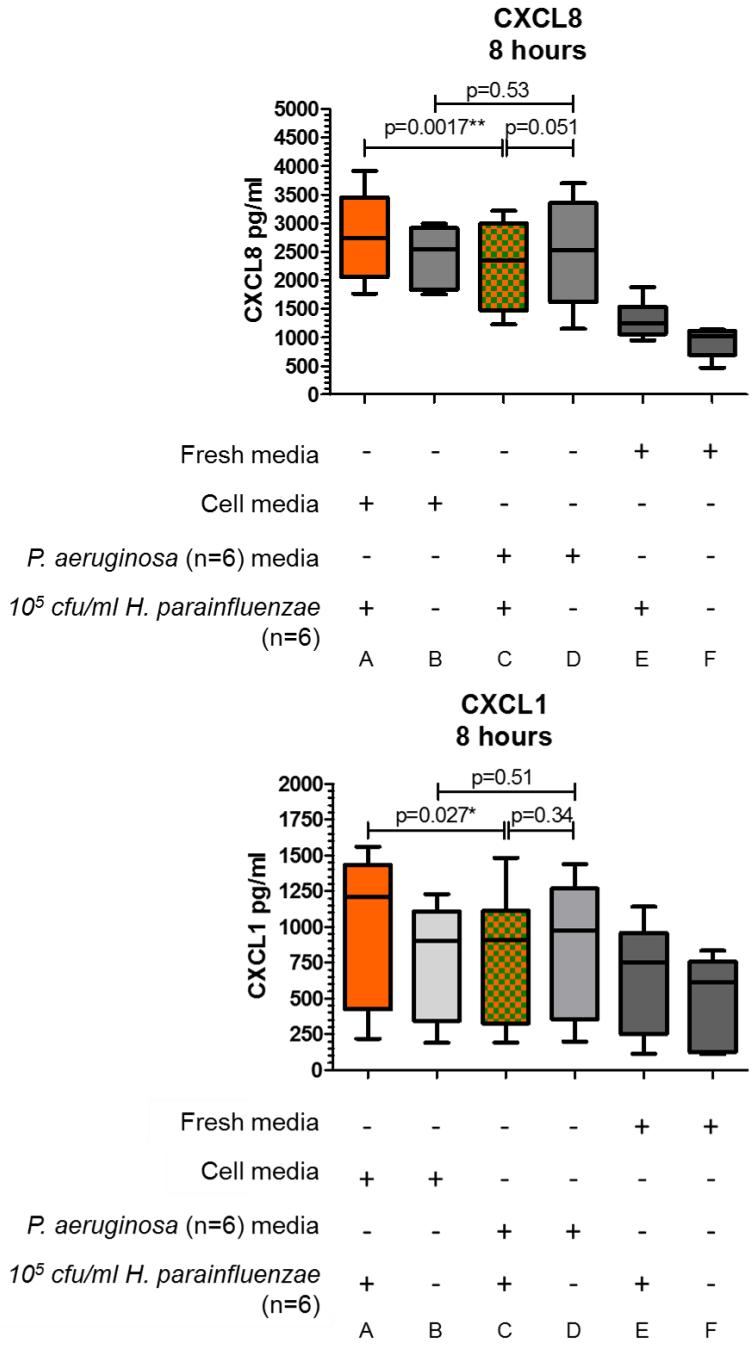


Figure 61. CXCL8 and CXCL1 production from 16HBE cells after cultured with *H. parainfluenzae* in *P. aeruginosa* media for 8 hours. 16HBE cells were cultured with 10^5 cfu/mL *H. parainfluenzae* (n=6) in different media for 8 hours. After 8 hours, media was then collected. Media CXCL1 and CXCL8 levels were measured by ELISA. Data are presented in box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8 and CXCL1.

3.5.8 *P. aeruginosa* co-cultured with 16HBE cells with media that had cultured cells and *H. parainfluenzae*

We also co-cultured 1×10^5 cfu/mL *P. aeruginosa* and 16HBE cells in media that had cultured 16HBE cells and *H. parainfluenzae* (*H. parainfluenzae* media), show as column B in Figure 56.

P. aeruginosa and 16HBE cells were also cultured in media that has cultured only 16HBE cells (cell media) as a comparison, show as column A in Figure 62.

Control group was 16HBE cells cultured with 1×10^5 cfu/mL *P. aeruginosa* in fresh media, show as column C in Figure 62.

From CXCL8 and CXCL1 results, there was no significant difference between 16HBE cells and *P. aeruginosa* that were cultured in *H. parainfluenzae* media compare to the group that was cultured in cell media(CXCL8 p=0.4, CXCL1 p=0.7).

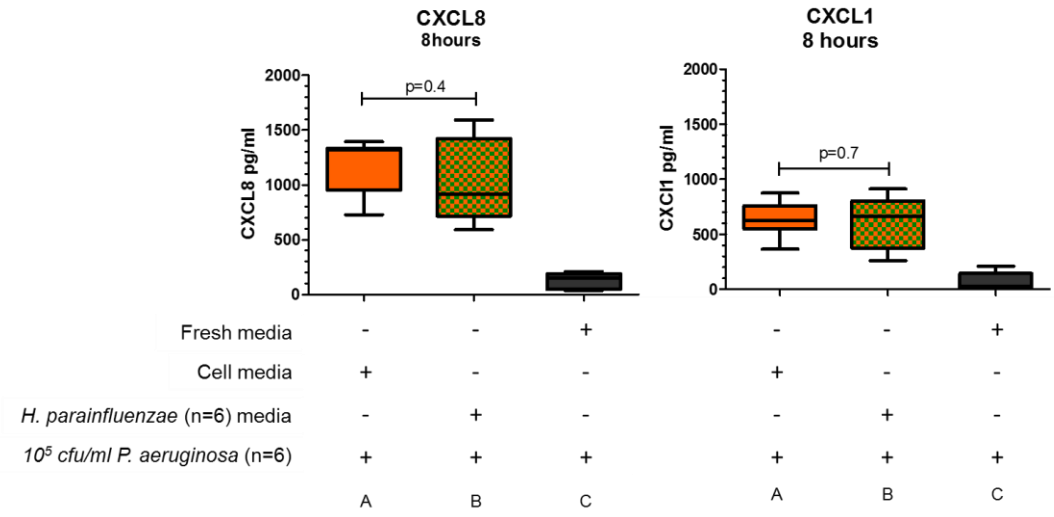


Figure 62. CXCL8 and CXCL1 production from 16HBE cells after cultured with *P. aeruginosa* in *H. parainfluenzae* media for 8 hours. 16HBE cells were cultured with 1×10^5 cfu/mL *P. aeruginosa* (n=6) in different media for 8 hours. After 8 hours, media was then collected. Media CXCL1and CXCL8 levels were measured by ELISA. Data are presented in box and whisker plots. The box shows +/- IQR. The line inside the box is median. P-value was calculated by paired t-test. Y axis shows the actual value of CXCL8 and CXCL1.

3.5.9 *H. parainfluenzae* cultured with media that had cultured 16HBE cells and *P. aeruginosa* on chocolate blood agar plate

Previously results showed that the media that had cultured *P. aeruginosa* and 16HBE cells can significantly reduce 16HBE cells inflammatory responses to *H. parainfluenzae*. Since bacterial load is an important factor to determine the inflammatory response, we proposed that this media may reduce *H. parainfluenzae* growth then reduce 16HBE cells inflammatory responses. As a positive control, *H. parainfluenzae* (n=5) were plated with 100 µL 15 µg/mL polymyxin B, which has been tested to inhibit the growth of *H. parainfluenzae*. The result shows that the media had cultured *P. aeruginosa* and 16HBE cells did not reduce *H. parainfluenzae* growth. (Figure 63)

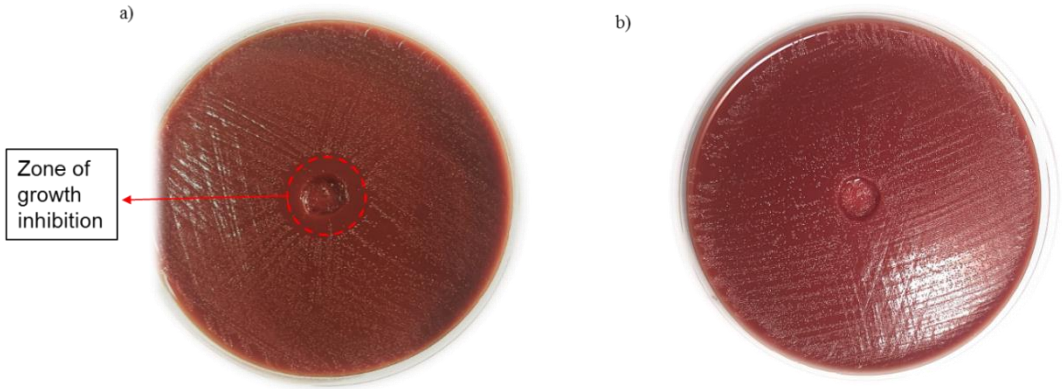


Figure 63. ***H. parainfluenzae* cultured on CBA agar with *P. aeruginosa* media.** a) *H. parainfluenzae* cultured with 15 µg/mL polymyxin B on CBA plate overnight (n=5) showing zone of inhibition. b) *H. parainfluenzae* cultured with the media that had cultured *P. aeruginosa* and 16HBE cells on CBA plate overnight (n=5) did not show a zone of inhibition.

3.5.10 *H. parainfluenzae* and 16HBE cells cultured with media that had cultured 16HBE cells and *P. aeruginosa*

Although *P. aeruginosa* and 16HBE cells did not significantly reduce *H. parainfluenzae* growth on CBA plates from the last experiment, it could be that this process requires the presence of 16HBE cells. So 16HBE cells were again co-cultured with *H. parainfluenzae* (n=5) in the media that had cultured 16HBE cells and *P. aeruginosa* (n=5). After 8 hours, the supernatant was collected and diluted in one time and 100 times, then 100 µL of the diluted supernatant was plated on CBA plates. Colony number was calculated the next day (Figure 64).

Surprisingly we found that *H. parainfluenzae* that was co-cultured with media that had cultured *P. aeruginosa* and 16HBE cells showed a significantly higher bacterial load compared to *H. parainfluenzae* that was co-cultured with media that had only cultured 16HBE cells (Figure 58). Instead of a suppressing effect, there was a promoting effect from *P. aeruginosa* to *H. parainfluenzae* bacterial growth. The median bacterial load for *H. parainfluenzae* that cultured in cell media was 1.75×10^4 cfu/mL, for *H. parainfluenzae* that cultured in *P. aeruginosa* media was 1.18×10^5 cfu/mL.

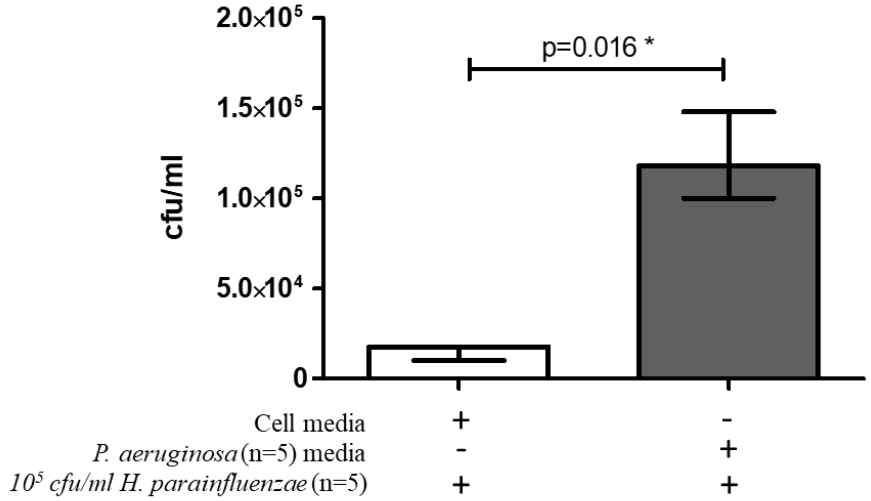


Figure 64. **Bacterial load of *H. parainfluenzae* after co-cultured with 16HBE cells in *P. aeruginosa* media.** *H. parainfluenzae* (n=5) was co-cultured with 16HBE cells for 8 hours with media that had cultured 16HBE cells and *P. aeruginosa* (*P. aeruginosa* media) or media that had cultured only 16HBE cells (cell media). After 8 hours, supernatant was collected and diluted 1 time, 10 times and 100 times. 100 µL of diluted solution was plated on CBA plate, plates were incubated overnight, and colony number was calculated next day. Y axis shows the bacterial load that was calculated from colony number. P-value was calculated using paired t-test.

3.5.11 *H. parainfluenzae* and 16HBE cells cultured with broth that had cultured *P. aeruginosa*

The media that had cultured *P. aeruginosa* and 16HBE cells can promote the growth of *H. parainfluenzae*, to figure out whether 16HBE cells is essential for this phenotype, *H. parainfluenzae* (n=5) was co-cultured with 16HBE cells with the broth that had only cultured *P. aeruginosa* (n=5).

After 8 hours, there was still a significant increase in the bacterial load of the *H. parainfluenzae* that were cultured with *P. aeruginosa* broth (p=0.048). This result suggests that *P. aeruginosa* can promote the growth of *H. parainfluenzae*. The median bacterial load for *H. parainfluenzae* that were cultured without *P. aeruginosa* broth was 8.75×10^5 cfu/mL, for *H. parainfluenzae* that were cultured with *P. aeruginosa* broth was 7.58×10^6 cfu/mL (Figure 65).

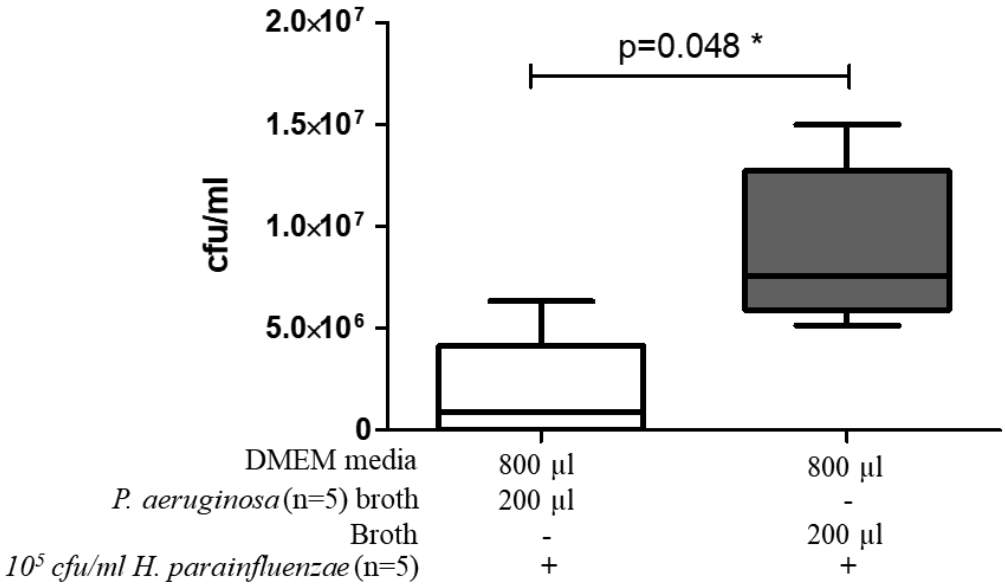


Figure 65. **Bacterial load of *H. parainfluenzae* after co-cultured with 16HBE cells in *P. aeruginosa* broth.** *H. parainfluenzae* (n=5) was co-cultured with 16HBE cells for 8 hours with 800 µL DMEM media and 200 µL filtered BHI broth that had cultured *P. aeruginosa* (*P. aeruginosa* broth). As comparison, *H. parainfluenzae* (n=5) was co-cultured with 16HBE cells for 8 hours with 800 µL DMEM media and 200 µL BHI broth. After 8 hours, supernatant was collected and diluted in 1 time, 10 times and 100 times. 100 µL of diluted solution was plated on CBA plate, plates were incubated overnight, and colony number was calculated next day. Y axis shows the bacterial load that was calculated from colony number. P-value was calculated using paired t-test.

3.5.12 *H. parainfluenzae* cultured with broth that cultured *P. aeruginosa*

Finally, *H. parainfluenzae* (n=5) was cultured with broth that had cultured *P. aeruginosa* (n=5). Bacterial load (OD value) was measured at different time points (2 hours, 4 hours, 6 hours, 8 hours and 24 hours). (Figure 66)

The broth that had cultured *P. aeruginosa* significantly increased *H. parainfluenzae* bacterial load at 6 hours, 8 hours and 24 hours compared to the *H. parainfluenzae* that were cultured only in broth (p=0.02, p=0.009 and p=0.02 respectively).

The median OD value for the *H. parainfluenzae* that were grown in the filtered broth which had previously cultured *P. aeruginosa* was 0.107 at 2 hours, 0.191 at 4 hours, 0.253 at 6 hours, 0.408 at 8 hours, 0.749 at 24 hours. The median OD value for *H. parainfluenzae* that were grown in the normal broth was 0.102 at 2 hours, 0.146 at 4 hours, 0.177 at 6 hours, 0.240 at 8 hours and 0.531 at 24 hours. (Figure 66)

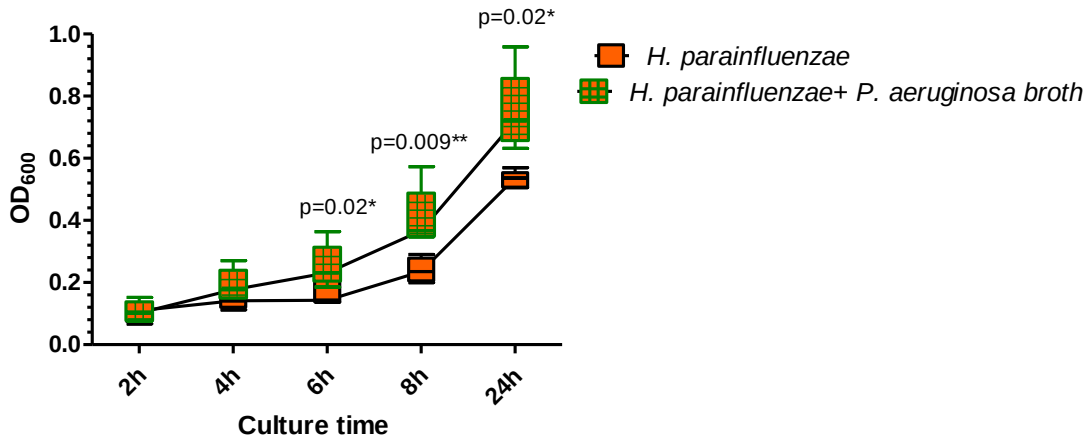


Figure 66. **Bacterial load of *H. parainfluenzae* after co-cultured with *P. aeruginosa* broth.** *H. parainfluenzae* (n=5) were incubated with 4 mL BHI broth and 1 mL filtered BHI broth that cultured *P. aeruginosa.* In control group, *H. parainfluenzae* were cultured with 5 mL BHI broth. After 2hours, 4 hours, 6 hours, 8 hours and 24 hours, OD value was measured at each time points. Y axis shows the OD value. P-values were calculated by paired t-test.

3.5.13 Conclusion for aim 5

- Poly-microbial (*H. parainfluenzae* and *P. aeruginosa*) infection reduced 16HBE cell inflammatory responses (CXCL8, CXCL1, LCN2 production) compared to single bacterial infection at 10^5 cfu/mL
- *P. aeruginosa* media at 8 hours can reduce the inflammatory responses caused by *H. parainfluenzae* on 16HBE cells, but *H. parainfluenzae* media could not affect the inflammatory response caused by *P. aeruginosa* on 16HBE cells.
- *P. aeruginosa* media can promote the growth of *H. parainfluenzae* rather than inhibit its growth.
- Further research is planned to determine what components of the *P. aeruginosa* media may be related to the inhibition effect.

Chapter 4

Discussion

Chapter 4: Discussion

4.1 Discussion for hypothesis 1

4.1.1 *H. parainfluenzae* are common in bronchiectasis

H. parainfluenzae are often treated as a commensal bacterium in lower airway diseases. One of the main reasons is that in the lower airways *H. parainfluenzae* are not as prevalent as *H. influenzae*. For example, in CF, before 2000, the number of *H. parainfluenzae* detected in human lower airways was about a third of the number of *H. influenzae*. However, the number of *H. parainfluenzae* had significantly increased by 2009. By 2012, *H. parainfluenzae* was almost three times that of *H. influenzae*. This significant increase could be due to the development of better techniques for *H. parainfluenzae* culture and identification, as well as increased awareness of *H. parainfluenzae*. (Ebbing and Robertson, 2015)

In this study, the number of patients that presented *H. parainfluenzae* was almost equal to those with *H. influenzae*. There was no *H. parainfluenzae* reported from the NHS microbiology laboratory. There could be a couple of reasons: 1) the technique to isolate and identify bacteria from sputum samples. *Haemophilus* species are fastidious bacteria to culture; In the NHS laboratory, usually, a loop (≈10 μL) of liquefied sputum sample was used for bacterial culture. In my microbiology method, liquefied sputum was diluted 100 times and 10000 times first, then extract 100 μL of each diluted sputum for bacterial culture. In the NHS microbiology method, a loop of liquefied sputum was tiny volume and may lead to a selective representation of bacteria in sputum. Furthermore, In the NHS microbiology method, the liquefied samples were not diluted, which may results in less *Haemophilus* species growth than the sputum actually contains, since *Haemophilus* species can be overwhelmed by other species when the growing condition is crowded 2) in the clinical practice, *H. parainfluenzae* are often considered as a commensal bacteria, so even if the NHS microbiology laboratory could identify *H. parainfluenzae* from a sputum sample, it may not be reported. This could be another key reason that no *H. parainfluenzae* was reported from NHS microbiology laboratory.

No capsulated *H. influenzae* have been recovered from this patient's cohort. NTHi have been reported more frequently from lower airways diseases than capsulated *H. influenzae* because of the use of Hib vaccination. My study confirmed this in bronchiectasis as well. For the identification of NTHi from capsulated *H. influenzae*, gene *bexA* had been commonly used. The polysaccharide capsule of typeable *H. influenzae* strains is encoded by the *cap* locus, which is composed of three distinct regions: regions I to III. The genes contained within regions I and III, designated *bexDCBA* and *hcsAB*, respectively, are highly conserved across all capsular types and are required for transport of capsule constituents across the outer membrane (Satola *et al*, 2003). The use of *bexA* gene to identify NTHi sometimes failed to identify capsule-deficient variants, so the use of gene *bexB* was proposed, there was no reported *bexB* partial deletions analogous to those observed in *bexA*. The combined use of *bexA* and *bexB* gene significantly increased the accuracy of identification. (Gregg S *et al*. 2011)

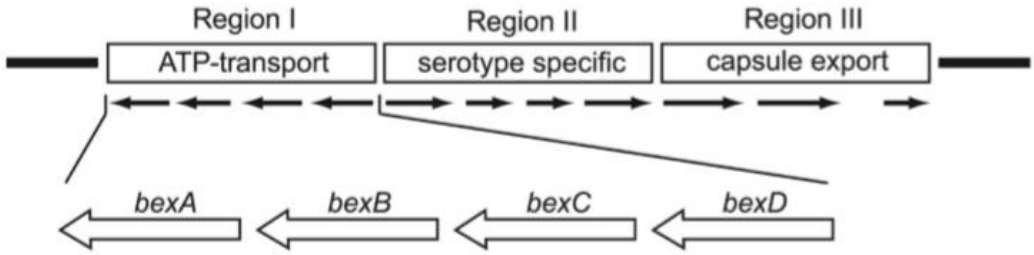


Figure 66. **The three *cap* locus in *H. influenzae*.** The cap locus is divided into three regions: I, II, and III; both *bexA* and *bexB* are located within region I. (Gregg S *et al*. 2011)

In our laboratory compared to NHS microbiology laboratory, we found fewer patients had MNF, instead more pathogens were identified. In our method, we serially diluted sputum samples and plated them at different dilutions, this contributes to the growth of the bacteria that grow in a low speed or in low bacterial load. Also, our method includes multiple molecular strategies (PCR and qPCR), which allows more specific identification of bacteria to minimize the chance of misidentification of pathogen.

In this patient cohort 5.6% (n=8) patients had more than one pathogen, this number is much smaller than the 208 patients cohort which was 19%. This could be due that fact that this group of patients was selected to only have *H. influenzae* as their main pathogen according to their latest NHS reports.

Limitations

The identification of *H. parainfluenzae* from *H. influenzae* was done by XV text in this study, which is the golden standard test for *H. parainfluenzae* identification. The experiment may have been enhanced if molecular identification methods were also used.

Haemophilus species are delicate bacteria when culture in the laboratory practice especially for storage. The PCR and qPCR tests were performed on the *Haemophilus* species that were recovered from -80° C, there was around 15% to 25% of bacteria could not grow successfully. This could be avoided if PCR and qPCR tests were performed as soon as bacteria were isolated from patients' sputum samples. However, the patients' samples were collected at different times through this study, it was not practical to run PCR or qPCR tests on a small number of samples, so, we decided to freeze and store the bacterial isolates until there was sufficient number of samples for PCR tests.

In this study, we only collected sputum sample once from each patient, so, the results cannot clarify if these patients are chronically colonised by *H. parainfluenzae*. For future study, it would be important to investigate if bronchiectasis patients would be chronically colonised by *H. parainfluenzae*.

4.1.2 *H. parainfluenzae* from the lower airways and upper airways are genetically different but related. They also trigger different inflammatory responses

H. parainfluenzae are very common upper airways bacteria in healthy people and bronchiectasis patients (Kosikowska *et al.*, 2016). *H. parainfluenzae* can colonise our upper airways and behave as commensal bacteria, it seems to suggest that *H. parainfluenzae* are not pathogenic (Bottone and Zhang, 1995).

In this study, *H. parainfluenzae* from same patient's lower airways and upper airways had different but related genomic profiles. The different but related genetic profile may suggest that the *H. parainfluenzae* from upper airways and lower airways were related, and some mutations may have happened or selected when *H. parainfluenzae* travelled from upper airways to lower airways due to the different environment. Furthermore, upper airways' *H. parainfluenzae* stimulated significantly higher inflammatory responses than the lower airways ones. The low inflammatory response from lower airways may contribute to the survival and colonisation of *H. parainfluenzae* in the lower airways.

This part of the project suggests that *H. parainfluenzae* from lower airways are different from those in the upper airways, so the fact that *H. parainfluenzae* do not trigger a host immune response in the upper airways does not mean that *H. parainfluenzae* are not pathogenic in bronchiectasis lower airways.

Limitations

In this part of the project, the sample size was small because of the difficulty of growing *H. parainfluenzae* from frozen stock. A more significant sample number could help to strengthen this result. Also, whole genome sequencing could be used to give better comparison between *H. parainfluenzae* from upper airways and lower airways.

4.1.3 *H. parainfluenzae* patients and NTHi patients have similar clinical symptoms

Pathogen infection usually adds to airways inflammation in bronchiectasis. To explore the pathogenicity of *H. parainfluenzae*, *H. influenzae*, a commonly accepted pathogen in bronchiectasis, was used as a comparison. In this study, serum WCC, ESR, CRP and ICAM-1 were measured. WCC is a basic clinical test marker for infection and inflammation, in bronchiectasis, patients usually have high number of neutrophils (Belda *et al*, 2000). ESR and CRP are both general inflammation markers. It has been found that bronchiectasis patients have higher serum WCC, ESR and CRP compared to healthy people (Ergan Arsava and Coplu, 2011), and in patients that are colonised with pathogens compared to the patients that had MNF, there was also higher serum inflammation (Chalmers, J. D. *et al*. 2012). ICAM-1 mediates the interaction between the neutrophils and bronchial epithelial cells, it can be produced from epithelium and has been demonstrated to be upregulated in bronchiectasis (Chan SC *et al*, 2008). For sputum inflammation, MPO was measured, it is the most abundant pro-inflammatory enzyme stored in the neutrophilic granulocytes, it can reflect the activation of neutrophils. In bronchiectasis, there is higher MPO levels in colonised patients compared to the non-colonised patients (Pulli *et al*, 2013). Besides the inflammatory markers, we also compared the patients for their BSI scores. It takes into consideration of multiple clinical factors, is a useful predictor of morbidity and mortality, it can also give predictions of hospital admission, exacerbation and life quality (Chalmers *et al*, 2014). The use of these markers as well as patient's lung function tests can give a complete comparison for the clinical relevance of NTHi and *H. parainfluenzae* in bronchiectasis.

From this study, patients that had *H. parainfluenzae* did not show a significant difference in their serum and sputum inflammation markers from those patients that had *H. influenzae*. This suggests that *H. parainfluenzae* may have similar clinical relevance as *H. influenzae* in bronchiectasis.

H. parainfluenzae patients had significantly lower bacterial load than *H. influenzae* patients (Figure 24). This may suggest that *H. parainfluenzae* are less efficient than *H. influenzae* at persisting in lower airways. In those *H. parainfluenzae* patients, more

than half of them had bacterial loads of more than 10^6 cfu/mL. In bronchiectasis, when patients' sputum bacterial load is 10^6 cfu/mL or more, they usually have significantly increased serum and sputum inflammation, also more frequent out-patient exacerbations (Chalmers, J. D. *et al.* 2012).

Patients that had higher bacterial load of *H. parainfluenzae* had significantly higher serum ESR, ICAM-1 and sputum MPO in comparison to the patients that had lower bacterial load of *H. parainfluenzae*. The high bacterial load is thus necessary for *H. parainfluenzae* to be pathogenic in bronchiectasis.

Limitations

In this study, blood and sputum samples were collected from patients only for a single time, but patient's clinical symptoms can be influenced by a lot of other factors, especially patients also with COPD, asthma or some other clinical conditions. Multiple time points may be better for this case. However, since sputum microbiology test was only performed once for each patient as previously mentioned, the inflammatory data from other time points may relate to different microbiology results, so in this study, only one time point (the same time as sputum microbiology test was done) was chosen. This study would be continued by comparing a larger cohort of patients with bronchiectasis and chronically colonised with *H. parainfluenzae* or NTHi.

Also, we compared the patients that had both serum sample and sputum sample. Due to the fact that all patients were at stable stage, their sputum sample volume was limited, so not all the patients gave sufficient volume of sputum sample for bacteria identification and also MPO measurement. The sample size was smaller in this part of study compared to the number of patients that we got microbiology results.

There are other ways to assess the severity of bronchiectasis, for example the FACED score, which compare to BSI score, is an easier to use tool but incorporate less dichotomic variables. Study had shown these two scores gave similar results and both can be used for routine clinical work (Minov J *et al*, 2015). There is also the St. George's Respiratory questionnaire, the Leicester Cough questionnaire, the Bronchiectasis Health questionnaire and the incremental shuttle walk test, those are

all found to be useful clinical end points and can be included in future study (Cartlidge MK *et al*, 2018).

4.1.4 *H. parainfluenzae* triggers similar inflammatory responses from epithelial cells as *H. influenzae*

4.1.4.1 Cell culture

In this study, three different epithelial cells were used. 16HBE is a widely used cell line as a model for the airway's epithelium barrier function. They can express cytokines and form tight junction. In this study, cytokine production was the main target. NHBE are normal human primary bronchial cells; they were isolated from the epithelial lining of airways above the bifurcation of the lungs. Compared to 16HBE cells, NHBE cells, as primary cells, can represent more normal epithelial cells' behaviour. However, NHBE cells are taken from healthy people, and in bronchiectasis, patients have damaged airways and a deficient immune system, so patients' nasal epithelial cells were also isolated and tested. We in total recruited 10 stable bronchiectasis patients and collected their nasal brush for cell cultural. Five nasal samples successfully grown in the laboratory, four samples could not grow due to contamination, one sample stopped growing after one passage.

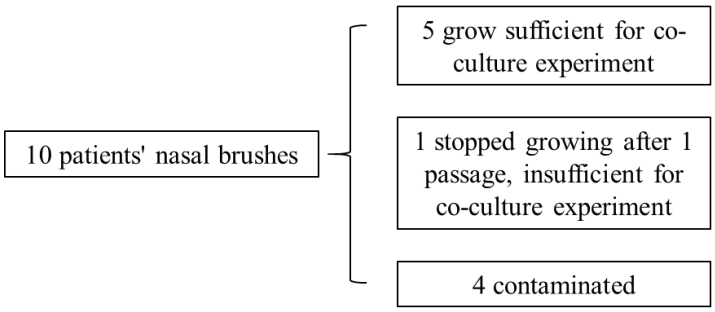


Figure 67. **The growth of patients nasal epithelial cells**

Limitations

In this study, we have included three different cells, especially the patients' nasal epithelial cells. However, we did not use patients' bronchial epithelial cells. This could be achieved by culturing bronchial tissue obtained from patients (Yaghi A1, Zaman A and Dolovich M, 2010). This is an invasive procedure for the patients, also more time consuming and complex. It could be included for future study since we have confirmed

H. parainfluenzae can cause inflammatory responses from nasal epithelial cells, it would be important to also prove that *H. parainfluenzae* can trigger similar inflammatory responses from patients' lower airways epithelial cells.

The effect from patient's macrophage and neutrophils on *H. parainfluenzae* is important as well. From this study, we found that patients had *H. parainfluenzae* had smaller bacterial load compared to NTHi. It is interesting to see if phagocytosis to *H. parainfluenzae* from patients' neutrophils would be more efficient to NTHi, which could be the reason for the smaller number of *H. parainfluenzae* in bronchiectasis patients.

Animal tissue model had been involved in this study. We isolated a 1mm×1mm size bronchial tissue from healthy porcine model. Then we co-cultured this bronchial tissue with *H. parainfluenzae*, NTHi and *P. aeruginosa* for different hours. The cultural media was collected and cytokine production was measured. We did not observe significant cytokine production and there was insufficient number of tissues, so this part of study was stopped without further input. There is also no bronchiectasis animal model available yet. On animal model we can mimic long term bacterial colonisation, which can help to understand the long-term effect of *H. parainfluenzae* on epithelium. Research about bronchiectasis animal model would be very appreciated.

4.1.4.2 Cytokines production were time-dependent

To figure out the optimum time point to investigate cell inflammatory response to *H. parainfluenzae*, bacteria were co-cultured with 16HBE cells for 1 hour, 2 hours, 4 hours,6 hours, 8 hours and 24 hours. For *H. parainfluenzae* and NTHi, after 8 hours CXCL8 production reached to peak, no significant difference was observed between 8 hours and 24 hours. And from the LDH assay, we found that after 16HBE cells co-cultured with *H. parainfluenzae* after 24 hours, there was significant cell injury or death. According to these, 8 hours was the best time point for the purpose of this study when there was sufficient cytokine production from cells and the cells were still at a good condition.

Limitations

For different cells and cytokines, there were differences in the hour when the cytokine production reached to the peak. For NHBE cells, CXCL1 production triggered by all three bacteria was in peak at 6 hours rather than 8 hours. For the further study, 8 hours was still used for compare the CXCL1 production triggered by different bacterial load of *H. parainfluenzae*. This may lead to less significant change between low bacterial load group and high bacterial load group.

4.1.4.3 Cytokines produced by epithelial cells

Epithelial cells play a crucial role in host defence against infection. Airways epithelium can recognise microorganisms by toll-like receptors (TLR). TLR2 and TLR4 can bind to bacterial LPS and then activate downstream pathways (Miller, Ernst and Bader, 2005). Through those pathways, epithelial cells can produce inflammatory mediators, chemotactic mediators and antimicrobial substances. In this study, mainly inflammatory and chemotactic mediators were measured. These mediators can initiate an inflammatory reaction and recruit phagocytosis. CXCL8, CXCL1 and ICAM-1 all can contribute to neutrophil migration(Mukaida, 2015; Filippo *et al.*, 2018; Yang *et al.*, 2005), CXCL1 also give positive feedback to MAPK/ERK pathway (Physiology, 2014), NFκB production(Papa *et al.*, 2004) and interleukin 17 production (Zhao *et al.*, 2014), which can promote cytokine production. LCN2 can directly inhibit bacterial growth by interrupting their iron pathway (Lois *et al.*, 2015; Kumar *et al.*, 2015). From cytokine array results, *H. parainfluenzae* can also stimulate strong production of cytokines like GM-CSF, which is important for macrophage differentiation and activation (Shibata *et al.*, 2001; Berclaz *et al.*, 2007)(Shibata *et al.*, 2001; Berclaz *et al.*, 2007); IL6, can contribute to CRP production, a key marker for inflammation in the airways (Hashizume, Hayakawa and Suzuki, 2009; Tanaka, Narazaki and Kishimoto, 2014); TNFα, can add to CRP levels and neutrophil migration (Eonard *et al.*, 1999). All these may indicate that *H. parainfluenzae* can lead to a strong inflammatory response from epithelial cells.

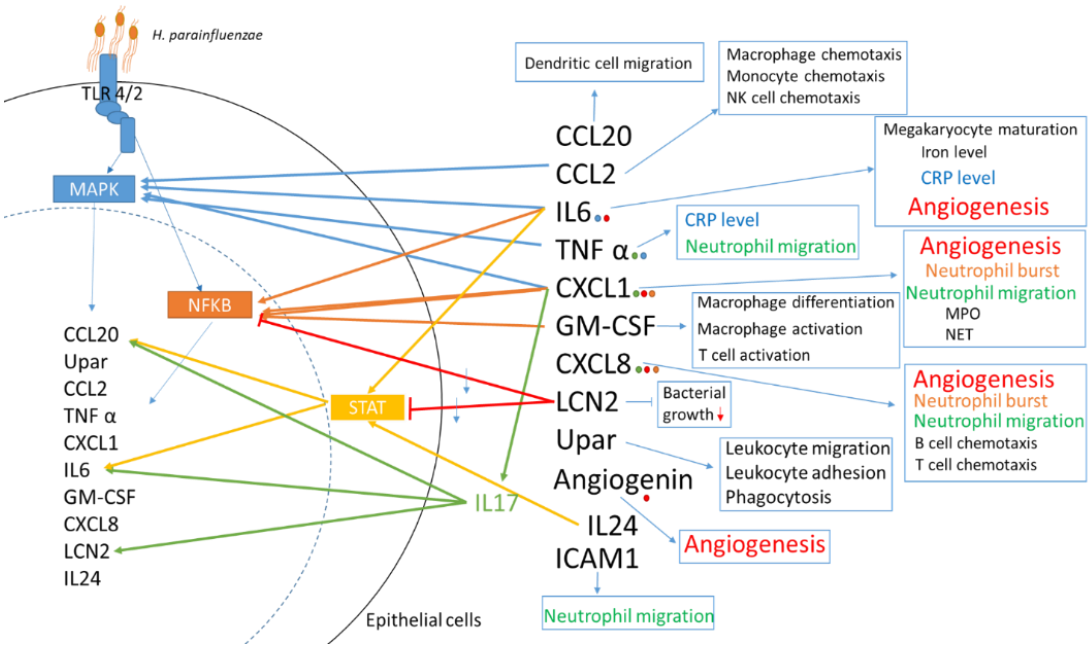


Figure 68. The cytokines that were up regulated by *H. parainfluenzae* on 16HBE cells. *H. parainfluenzae* can activate the production of CCL20, CCL2, IL6, TNF α, CXCL1, GM-CSF, CXCL8, LCN2, Upar, Angiogenin, IL24 and ICAM-1. Those cytokines are related to multiple inflammatory responses.

4.1.4.4 Inflammatory response caused by *H. parainfluenzae* is similar to *H. influenzae*

The main finding was that *H. parainfluenzae* could induce a similar epithelial immune response as *H. influenzae*. *H. influenzae* and *H. parainfluenzae* are both gram-negative bacteria; LOS is their main virulence factor. It seems evident that they can cause a similar response from the cell. However, LPS differs from bacteria to bacteria. Such as *H. parainfluenzae* and *Prevotella melaninogenica* (both gram-negative) stimulate a significantly different inflammatory response from macrophages, it is mainly because of their different LPS structure. *H. parainfluenzae* have a short acyl chain LPS while *P. melaninogenica* has a long acyl chain LPS (Goleva *et al.*, 2013). Between *H. influenzae* and *H. parainfluenzae*, their LOS is similar (Young and Hood, 2013), but, not identical. Compared to *H. influenzae*, *H. parainfluenzae* have a reduced capacity for synthesis of LOS outer core structure, which may lead to a lack of phase variation of *H. parainfluenzae* LOS molecule (Young and Hood, 2013). At the same time, *H. parainfluenzae* LOS contains O-antigen, which has not been found in *H. influenzae*.

O-antigen facilitates *H. parainfluenzae* survival from the human serum killing effect(Young *et al.*, 2013)(Vitiazeva *et al.*, 2011).

H. parainfluenzae have also been reported to have capsule structures, while all the *H. influenzae* found from this cohort were all NTHi, which do not have a capsule. The capsule is another critical virulence factor. It can protect bacteria from been recognised and engulfed by immune cells, and also help bacteria to attach to different surfaces (Moxon and Kroll, 1990).

Knowing the differences between these two *Haemophilus* species, it was essential to specifically investigate the pathogenicity of *H. parainfluenzae in vitro*. From the cell infection experiments, *H. parainfluenzae* at high bacterial loads can stimulate significant epithelial cell cytokine production and cell injury or death. This suggests that *H. parainfluenzae* can be a potential pathogen in bronchiectasis, although further research needed.

4.1.4.5 Epithelial cells response to *P. aeruginosa*

In the cell culture experiment, it has been noticed that after 24 hours, *P. aeruginosa* can trigger significantly higher CXCL8 production than the two *Haemophilus* species. *P. aeruginosa* not only have LPS as a virulence factor, but also produce toxins such as PCN(Lau *et al.*, 2004). The bacterial load of *P. aeruginosa* were significantly higher than *H. parainfluenzae* and NTHi after 8 hours and 24 hours growth, and these could also be the reason for the high CXCL8 level at 24 hours.

Limitations for 4.1.4.3, 4.1.4.4 and 4.1.4.5

For these parts we found that *H. parainfluenzae* can trigger significant inflammatory responses from epithelial cells by measuring cytokine productions. There are other measurements can be used to investigate the virulence of *H. parainfluenzae* on epithelial cells. For example, *H. influenzae* have been shown to be able to enter epithelial cells using electron and confocal microscopic analysis (Sajjan *et al*, 2008), which is not shown for *H. parainfluenzae*. It would be also important to see if *H. parainfluenzae* can disrupts the barrier function of airway epithelial cells.

In those epithelial cell co-culture experiments, we co-cultured epithelial cells with multiple strains of *H. parainfluenzae*, NTHi and *P. aeruginosa*. The addition of LPS positive control would have strengthened these results, and further experiments to block LPS while co-culture with epithelial cells would complement the heat-kill bacteria experiment to support the theory that LPS is the main virulence factor of H. parainfluenzae. Previous experiment has attempted to use polymyxin B to block bacterial LPS, however it was also harmful to 16HBE cells, so a better LPS blocking reagent needs to be used.

4.1.5 Future work for hypothesis 1

Our data explored the pathogenicity of *H. parainfluenzae* in stable bronchiectasis patients. It is crucial to also investigate *H. parainfluenzae* in bronchiectasis exacerbation. That *H. parainfluenzae* are common in stable patients does not mean it would still be in exacerbation patients. If *H. parainfluenzae* can lead to bronchiectasis exacerbation. How frequent it would be in bronchiectasis patients? Would those patients still have similar serum and sputum inflammation as patients that have *H. influenzae* exacerbation? To explain the mechanism, the research could focus on: 1) Epithelial cells are the first line of defence in our immune system. In this study, we investigated the inflammatory response from epithelial cells. For future work, we can isolate neutrophils and macrophages from patients' blood and bronchoalveolar lavage samples to figure out if those immune cells will also give a significant response to *H. parainfluenzae*. 2) Besides causing an inflammatory response, another important factor for the pathogenicity of *H. parainfluenzae* are whether they can persist in the airways. Our previous results show that patients had *H. parainfluenzae* had a lower bacterial load; it could be related to a more efficient killing function from the immune system to *H. parainfluenzae*. For this, phagocytosis effect of neutrophils to *H. parainfluenzae* would be important to investigate.

4.2 Discussion for hypothesis 2 - *H. parainfluenzae* and *P. aeruginosa* together reduced the inflammatory response

From this study, when *H. parainfluenzae* was co-cultured with *P. aeruginosa*, there was an inhibitory effect on 16HBE cells inflammatory response. This inhibitory effect only appeared when bacteria were in low bacterial load (10^5 cfu/mL).

The human airways are colonised by millions of different bacteria. In bronchiectasis, it has been suggested that the sudden rise of bacterial load of one or a few bacteria could lead to an imbalance of the microbiome (Woo *et al.*, 2019), which contributes to exacerbations. This theory can be further supported by these results. When bacteria were all in a small dosage, they stimulated less inflammation from cells. Bacteria may form competitive or cooperative relationship, which results in a "peaceful" environment. However, when bacterial load increases, the relationship on longer exist, which may interrupt the balance and lead to an "un-peaceful" environment. (B.Rogers, 2018)

P. aeruginosa have already been known to inhibit the growth and biofilm formation of *Staphylococcus aureus* (Kim *et al.*, 2014); it may have the same inhibitory effect on *H. parainfluenzae*, after which the decreased number of bacteria may lead to a milder inflammatory response from 16HBE cells. Although, from the aspect of bacteria, persistence in the host is more relevant than triggering host immune response. This decrease in inflammatory response could also be a result of cooperation between the two bacteria to escape from being recognised and cleared by the host immune system. (Apollo Stacy, 2015)

After finding out that *H. parainfluenzae* and *P. aeruginosa* together reduced the inflammatory response from 16HBE cells, we then found that the media that had cultured *P. aeruginosa* could reduce the CXCL8 and CXCL1 production caused by *H. parainfluenzae*. In comparison, media that had cultured *H. parainfluenzae* could not do the same to *P. aeruginosa*. This shows that something produced by *P. aeruginosa* could work on *H. parainfluenzae* or the cells, which lead to a decrease of cytokine production. For this study, we looked into the interaction between *P. aeruginosa* media and *H. parainfluenzae*.

Although this part of the study is a preliminary attempt to explore bacterial interaction, there are still a lot of questions that remain unanswered, or I would rather say a lot of possibilities for future work.

Our results suggested a cooperative relationship between *P. aeruginosa* and *H. parainfluenzae*. *P. aeruginosa* turned out to be able to promote the growth of *H. parainfluenzae* rather than inhibit it; this leads to a huge question: How does this interaction results in the lower inflammatory response?

4.3 Future work

As previously mentioned, the reduction effect led by *P. aeruginosa* media could be because of interaction with bacteria, or with cells. From this study, this question remains unsolved. Although we found that *P. aeruginosa* can positively influence *H. parainfluenzae* growth, there is not enough evidence to prove that this was the reason for the change in the inflammatory response.

If the change of *H. parainfluenzae* growth was a key factor in this process, it is crucial to figure out what produced by *P. aeruginosa* that can interact with *H. parainfluenzae*. In bacterial interaction, there is a metabolic cross-feeding model, in which one species consumes the by-products of another. This may, therefore, facilitate *H. parainfluenzae* growth and change the proportion of the metabolic pathway of *H. parainfluenzae* or its by-products, eventually resulting in changes in its virulence.

If the change of *H. parainfluenzae* growth was not a key factor in this process, then it could be that something produced by *P. aeruginosa* can inhibit the inflammatory response. It has been observed that some mRNA released from *P. aeruginosa* outer membrane vesicles can negatively influence the production of CXCL8 from cells (Bomberger *et al.*, 2009). So far, from current results, figuring out what is in the *P. aeruginosa* media should be the next step to explore. Both metabolic or proteomic analysis would be a useful step for future research.

4.4 Final discussion

H. parainfluenzae as a successful commensal species, its potential role as a pathogen has long been neglected. Most studies about *Haemophilus* species were only focused on *H. influenzae*. *H. parainfluenzae* infection was considered too rare to merit any research and case reports, not only in bronchiectasis but also in other respiratory diseases.

In otitis media, *H. influenzae* have been found in 38% of 8198 patients (Broides et al., 2009). There is no clear data about the frequency of *H. parainfluenzae* in otitis media, however, a lot of research include *H. parainfluenzae* as a potential pathogen in otitis media, although there is no clear data to support this (Cardines et al., 2009).

In pneumonia, type b *H. influenzae* have been found to be the main *Haemophilus* species, they have caused 1304 cases of pneumonia per 100,000 children under five globally in 2000, with figures of 1724 for Africa and 283 for Europe (Watt et al., 2009). *H. parainfluenzae* are not commonly reported in pneumonia. Multiple group had reported isolation or growth of *H. parainfluenzae* in pneumonia, but the method used to classify *H. parainfluenzae* were not clear (Numazaki et al., 2004) (Pillai et al., 2000). There was case report about *H. parainfluenzae* causing pneumonia, but the prevalence of *H. parainfluenzae* is still unknown.

In COPD, *H. influenzae* have been considered as pathogen and contribute to 20-30% of COPD exacerbations and also frequent involved in stable COPD (Sethi and Murphy, 2008). *H. parainfluenzae* have been found to be frequently isolated from both stable and exacerbated COPD, but it has been thought not pathogenic (Taylor *et al*, 1992).

In this study, we established the prevalence of *H. parainfluenzae* in bronchiectasis, and also, we confirmed that *H. parainfluenzae* plays similar role as NTHi in stable bronchiectasis in patients' clinical symptoms and *in vitro* experiments. This will help our understanding about *H. parainfluenzae* in bronchiectasis, and further more may draw more attentions to *H. parainfluenzae* in different airway diseases.

A better understanding of *H. parainfluenzae* is also needed the paediatric bronchiectasis, in which *Haemophilus* species is more commonly detected (around 50%) (Kapur, N, 2012). There has been case report about *H. parainfluenzae* identified

from paediatric bronchiectasis patients (Pijnenburg et al., 2004), but information about *H. parainfluenzae* in this area is very limited. It is unknow the frequency of *H. parainfluenzae* in children with bronchiectasis.

Bacterial infection drives the airway inflammation and remodelling, and bacterial clearance can reduce markers of inflammation and improve clinical outcome in bronchiectasis (Murray et al., 2010). Therefore, the identification of *H. parainfluenzae* and the awareness of *H. parainfluenzae* can be a potential pathogen would benefit the patients with more efficient treatment. Currently in NHS laboratory, *H. parainfluenzae* are often dismissed as mixed normal flora. With more studies about the pathogenic role of *H. parainfluenzae*, the results may eventually reflect on the routine treatment for bronchiectasis.

Chapter 5

References

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